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Review

Protein kinetic stability

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ABSTRACT

The relevance of protein stability for biological function and molecular evolution is widely recognized. Protein stability, however, comes in two flavours: thermodynamic stability, which is related to a low amount of unfolded and partially-unfolded states in equilibrium with the native, functional protein; kinetic stability, which is related to a high free-energy barrier "separating" the native state from the non-functional forms (unfolded states, irreversibly-denatured protein). Such barrier may guarantee that the biological function of the protein is maintained, at least during a physiologically relevant time-scale, even if the native state is not thermodynamically stable with respect to non-functional forms. Kinetic stabilization is likely required in many cases, since proteins often work under conditions (harsh extracellular or crowded intracellular environments) in which deleterious alterations (proteolysis, aggregation, undesirable interactions with other macromolecular components) are prone to occur. Also, kinetic stability may provide a mechanism for the evolution of optimal functional properties. Furthermore, enhancement of kinetic stability is essential for many biotechnological applications of proteins. Despite all this, many published studies focus on thermodynamic stability, partly because it can be easily quantified *in vitro* for small model proteins and, also, because of the availability of computational algorithms to estimate mutation effects on thermodynamic stability. In this review, the opposite bias is purposely adopted: the experimental evidence supporting widespread kinetic stabilization of proteins is summarized, the role of natural selection in determining this feature is discussed, possible molecular mechanisms responsible for kinetic stability are described and the relation between kinetic destabilization and protein misfolding diseases is highlighted.

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1. Introduction

1.1. Thermodynamic stability versus kinetic stability

Usually, *in vitro* denaturation experiments aimed at determining protein stability are performed under simple solvent conditions, involve comparatively short time-scales and use small "model" protein systems. Operationally reversible denaturation is often found in these experiments and analysis of the denaturation profiles (i.e., the value of a suitable physical property versus temperature, concentration of a chemical denaturant, etc.) is generally carried out on the basis of equilibrium thermodynamics. In the simplest case, the data are found to conform acceptably to the two-state equilibrium model:

$$N \leftrightarrow U \tag{1}$$

where N is the native state and U is the unfolded state (actually an ensemble of more or less unfolded conformations) and K is the unfolding equilibrium constant,

$$K = \frac{[U]}{[N]} \tag{2}$$

which is related to the standard unfolding free-energy change [$\Delta G = G(\mathrm{U}) - G(\mathrm{N})$] through the Lewis equation,

$$\Delta G = -RT \cdot \ln K \tag{3}$$

Fit of the model to the experimental profiles ultimately leads to ΔG as a function of an environment parameter (temperature, concentration of a chemical denaturant). Extrapolation to "physiological" conditions (zero concentration of chemical denaturant, 37 °C, for instance), leads typically to a positive (although not too large) value of the unfolding free-energy change [1–3], indicating that $K<1$ and that the unfolding equilibrium is shifted towards the folded state. This result leads to the concept of protein thermodynamic stability: the biological function of the protein is guaranteed if equilibrium is established between the native and the unfolded (and partially unfolded) states of the protein and the unfolding thermodynamics favours the folded state under physiological conditions.

It must be recognized, nevertheless, that the biological function of the protein can be maintained, *at least during a certain physiologically relevant time-scale*, even if the native state is not thermodynamically stable with respect to non-functional states (which may include, not only unfolded and partially-unfolded states, but, also, irreversibly-denatured "states", such as aggregated or proteolyzed protein). All that is required is a sufficiently high free-energy barrier "separating" the native state from the non-functional forms. Assuming transition-state theory applies, the rate of irreversible denaturation is related to the free-energy barrier ($\Delta G^{\neq}$) through the Eyring equation:

$$k = k_0 \cdot \exp\left(-\Delta G^{\neq} \Big/ RT\right) \tag{4}$$

where k_0 is the front factor (for a discussion, see Ref. [4] and references quoted therein) and k is the rate constant for irreversible protein denaturation. If the activation free-energy value is large enough, the rate of irreversible denaturation will be low and the protein will remain in the native, functional state during a sufficiently large time-scale. In this case, we say that the protein is kinetically stable.

1.2. Two fundamental scenarios for protein kinetic stability

Protein kinetic stability is likely related to (at least) two different scenarios:

Scenario 1) The native, functional protein is thermodynamically stable with respect to unfolded and partially-unfolded states. However, these states can undergo irreversible alteration processes (aggregation, proteolysis, strong interactions with other momacromolecules, etc.) that lead to some kind of "final state" (unable to fold back to the native one). This scenario is summarized in the well-known Lumry–Eyring model:

$$N \Leftrightarrow U \Rightarrow F \tag{5}$$

where F is the final state [5–7]. Of course, modifications of the basic Lumry–Eyring model involving partially-unfolded states may be considered [6]. In the type of situation described by a Lurmy–Eyring model, the protein will eventually end-up in the non-functional, final state. Therefore, biological function in a physiologically relevant time-scale requires kinetic stabilization, i.e., a significant free-energy barrier along the way from N to F. The Lumry–Eyring model is important because irreversible alterations of unfolded or partially-unfolded states may be expected to occur efficiently in crowded and/or harsh, *in vivo* environments. The Lumry–Eyring model suggests, therefore, that natural selection has endowed many proteins with kinetic stability, even if this fact is not revealed in the "traditional" *in vitro* experiments [7].

Scenario 2) The native, functional protein is *not* thermodynamically stable with respect to unfolded and partially-unfolded states under physiological conditions. Biological function requires kinetic stabilization (a significant free-energy barrier between N and the unfolded or partially-unfolded states), even if the irreversible alterations discussed above (aggregation, proteolysis, etc.) do not readily occur. Of course, this second scenario poses a fundamental question: how did the protein arrive at the native state (starting from unfolded conformations) in the first place? We will return to this issue in Section 3, when we discuss the paradigmatic example of the α-lytic protease [8], but it can be advanced here that this enzyme is synthesized as a proenzyme containing a pro-region which provides efficient catalysis and thermodynamic driving force for folding and which is cleaved out upon completion of folding.

1.3. The relation between thermodynamic stability and kinetic stability

The two scenarios discussed in the preceding section immediately suggest several possible relationships between the thermodynamic and kinetic stabilities of a protein which range from a perfect correlation between the two properties to an absolute independence.

Assume a simple Lumry–Eyring scenario in which irreversible alterations of the unfolded state are not too fast, so that the U → F step (in the simple scheme of Eq. (5)) is rate-limiting. It can be easily shown [7,9] that, in this situation (corresponding perhaps to the protein under moderately harsh environment conditions) the rate constant for the overall irreversible denaturation process becomes the product of the unfolding equilibrium constant (Eq. (2)) times the rate constant for the U → F step. Under this "global unfolding" mechanism, effects on thermodynamic stability (mutation effects, for instance) will immediately affect kinetic stability. The idea that thermodynamic stability can, in some cases at least, translate directly into kinetic stability *in vivo* was already suggested by the early studies on intracellular protein degradation of Parsell and Sauer [10].

Assume now a Lumry–Eyring scenario in which the U → F step is very fast (corresponding to the protein under very harsh environment conditions) so that unfolding itself (N → U) becomes rate-limiting and the rate of irreversible denaturation equals the rate of unfolding [7,9]. In this situation, kinetic stability is determined by the free-energy barrier for unfolding and effects on thermodynamic stability may or may not translate into kinetic stability. For instance, according to a very simple view, the transition state for the N → U step would comprise unfolded regions, together with folded, native-like regions (see, however, Section 7.3 for a more realistic discussion). Mutations in those parts of the protein that become unfolded in the transition state are expected to show parallel effects on thermodynamic stability and kinetic stability (see, however, Section 5.2). In any case, it is clear that the free-energy barrier for unfolding may act as a "safety mechanism" that ensures significant kinetic even in harsh environments [7,9].

Finally, consider scenario 2 described above in which the native state is not thermodynamically stable with respect unfolded and partially-unfolded forms under physiological conditions and it is kinetically-trapped. In this case, thermodynamic stability and kinetic stability may be completely decoupled. Actually, it has been suggested [11] that this situation provides an efficient mechanism for the evolution of optimal functional properties, since the constraints imposed by the folding of thermodynamically stable proteins have been removed.

1.4. The relevance of kinetic stability: biological function in vivo and biotechnological implications

Irreversible alterations of proteins (proteolysis, aggregation, undesirable interactions with other macromolecular components) may easily take place in harsh extracellular conditions and in crowded intracellular environments. Even if these alterations occur from unfolded or partially-unfolded states, they will deplete the native, biologically-functional state in a time-dependent manner, as analyses of Lumry–Eyring models do show [6,7,12]. As a result, thermodynamic stability alone (i.e., a positive value of the unfolding free-energy change) does not guarantee that the protein will remain in the native state during the biologically-relevant time-scale. It follows that the biological function of many proteins *in vivo* is very likely linked to a significant degree of kinetic stabilization. Furthermore, kinetic stability may provide an efficient mechanism for the evolutionary optimization of functional properties [11], as we have noted above and will discuss in detail in Section 6.5. It has also been pointed out [11] that kinetic stability may function as a timer to regulate biological function in systems like serpins, membrane fusion proteins and heat shock transcription factor [13–18]. The possible biological relevance of kinetic stability in collagens has been discussed [19,20]. Finally, kinetic destabilization plays a fundamental role in many misfolding diseases, as we will elaborate in detail in Section 5.

In addition to the relevance *in vivo*, protein kinetic stability may be of considerable importance in *in vitro* biotechnological applications [21]. Researchers interested in fundamental, thermodynamic aspects of protein folding and stability may choose small "model" proteins and simple solvent conditions for which reversible, equilibrium folding/unfolding is observed. However, the proteins and environment conditions required for biotechnological applications will often result in irreversible denaturation and kinetic control of the operational stability. In fact, enhancing protein stability for biotechnological applications may in many cases mean improving some parameter related to kinetic stabilization [21]. A particularly relevant example is provided by the efforts (at both, the formulation and protein engineering levels) to ensure a suitable shelf-life of protein pharmaceuticals, such as monoclonal antibodies [22].

1.5. The outline of this review

A wide variety of experimental results provide indication of high free-energy barriers and likely kinetic stabilization of proteins: metastability of folded states and/or existence of alternative native or native-like states [23–28], marked hysteresis in folding/unfolding [29–31] and low unfolding rates [7,32,33] under physiological conditions (obtained, for instance, by extrapolation to zero denaturant concentration of the rate constant for urea- or guanidine-induced unfolding). Historically, however, early evidence for protein kinetic stability came from two kinds of studies: differential scanning calorimetry analyses on irreversible protein denaturation and experimental analyses on the stability in the α-lytic protease system. These studies are summarized in Sections 2 and 3, while recent approaches to the determination of kinetic stability in a proteomic scale are described in Section 4. The (growing) evidence that kinetic destabilization plays a fundamental role in the development of misfolding diseases is discussed in Section 6. It should become clear along this review that many protein systems are naturally-selected to have significant kinetic stability, but some particularly relevant studies that support natural selection of kinetic stability are emphasized in Section 5. Finally, our current (and rather imperfect) knowledge about the molecular mechanisms of kinetic stabilization is summarized in Section 7.

2. Early evidence for protein kinetic stability: differential scanning calorimetry of protein irreversible denaturation

2.1. A very brief introduction to differential scanning calorimetry

In the differential scanning calorimetry (DSC) approach to the determination of protein stability (for reviews, see [3,12,34–45]), a protein solution is heated at a constant temperature-scanning rate, heat capacity is measured as a function of temperature and

denaturation events are revealed by transitions ("heat capacity peaks") in the thermogram. Freire and Biltonen showed theoretically in 1978 [46] that equilibrium DSC thermograms are essentially equivalent to the relevant protein partition function and, therefore, contain all the relevant information about the temperature-induced denaturation process. In other words, analysis of equilibrium DSC thermograms should lead to the number of states involved in the denaturation process and the values of the relevant thermodynamic parameters for all these states. Indeed, DSC thermograms for complex protein systems have been commonly interpreted in terms of multi-state unfolding, often leading to a structural-domain description of the unfolding energetics [37]. More recently, the possibility of analyzing equilibrium DSC thermograms in terms of a continuum of states has been explored in relation with ultra-fast protein folding and the possibility of observing the global downhill regime [47–52].

2.2. The problem of the analysis of calorimetrically irreversible transitions

The analyses described above are based on equilibrium thermodynamics and require that the experimental DSC thermogram accurately reflects an equilibrium denaturation process [6,7,12]. In other words, chemical equilibrium must always be established between the protein states that are significantly populated during the DSC scan. The usual "equilibrium test" in DSC is the operational (or calorimetric) reversibility of the denaturation process: for the denaturation process to be considered reversible (or, strictly speaking, operationally reversible), a second (re-heating) scan, performed after cooling to room temperature, should yield a transition comparable to that in the first scan. Assuming that folding–unfolding is fast in the time-scale of the calorimetric experiment, operational reversibility provides some degree of support for the equilibrium character of the denaturation process responsible for the observed DSC transition. Unfortunately, protein thermal denaturation is often found to be calorimetrically irreversible, as no transition is detected in the re-heating run [6,7,12]. This suggests that a Lumry–Eyring kind of mechanism (Eq. (5)) applies, with some irreversible alteration step taking the protein to a final state unable to fold back to the native state. Indeed, it is a common occurrence in protein scanning calorimetry that the sample extracted from the calorimeter after completion of the experiment shows clear signs of aggregation. The analysis of DSC thermograms for irreversible protein denaturation is, in principle, an unclear issue, since the irreversible alteration step in a Lumry–Eyring scenario ($U \rightarrow F$ in Eq. (5)) is a purely kinetic process and, therefore, not amenable to an equilibrium thermodynamics description.

2.3. The two-state irreversible model

A significant advance in the experimental study of irreversible denaturation processes was achieved in the late eighties and early nineties, when it was realized that Lumry–Eyring kind of mechanisms reduce to a phenomenological, two-state kinetic process,

$$N \rightarrow F \tag{6}$$

provided that the irreversible alteration step ($U \rightarrow F$) is sufficiently fast to make negligible the population of intermediate states [6,12,53]. That is, the mechanism in Eq. (5) (Lumry–Eyring) is phenomenologically equivalent to that in Eq. (6) (two-state irreversible model) if the $U \rightarrow F$ step is fast, in such a way that any molecule U formed is immediately converted to F and, consequently, $[U] \ll [N] + [F]$ during the whole temperature range of the calorimetric transition. Actually, the two-state irreversible model can be seen as a limiting case of many different denaturation models that involve a kinetically-limited irreversible alteration step [6,7,12].

Due to its simplicity, the two-state irreversible model is amenable to straightforward, but rigorous mathematical analysis leading to simple equations that make precise predictions regarding the shape of the calorimetric transitions and their dependence with the scan rate of the calorimetric experiment (Fig. 1). The theoretical proposal of the two-state irreversible model was a milestone in the development of our understanding of protein stability, since it allowed researchers to make reliable tests of the possible kinetically-controlled character of the experimental thermal denaturation profiles. During the last 20 years, a surprising large number of *in vitro*, protein thermal denaturation processes (see, for instance, Refs. [4,21,53–109]) have been reported to conform qualitatively and, often, quantitatively to the two-state irreversible model or to closely-related kinetic models. A non-exhaustive list includes protein systems as diverse as triose phosphate isomerases [4], lipases [21,82], thermolysin [53], procarboxypeptidases and carboxypeptidases [54,58], hemocyanins [55,93], Ca^{2+}-ATPase from sarcoplasmic reticulum [56,84], membrane reconstituted yeast cytochrome *c* oxidase [57], phosphoglycerate kinase [59], β-lactamase [60], bacteriorhodopsin [61,104], actin [62,103], 5-enolpiruvoyl shikimate-3-phosphate synthase [63], the 8 kDa cytotoxin from the sea anemose *Radiantus macrodactillus* [64], bromelain [65], acetylcholinesterase [66], lactate dehydrogenase [67], lentil lectin [68,80], proteins in the Newcastle disease virus [69], F_0F_1 ATP synthase [70], proteins in erythrocyte ghosts [71], annexin [72], interferon gamma [73], uridine phosphorilase [74], mannitol permease [75], tubulin [76], chymopapain [77], mitochondrial F_1-ATPase [78], muscle creatine kinase [79], cry3A δ-endotoxin [81], glucoamylase [83], fibrinogen [85], rhodopsin and opsin [86,102], apolipoprotein C-1 [87], high-density lipoproteins [88,94,98], low-density lipoproteins [97], very-low-density lipoproteins [100], DNA polymerases [89], amylases [90,96], ovalbumin [91], glucose oxidase [92],

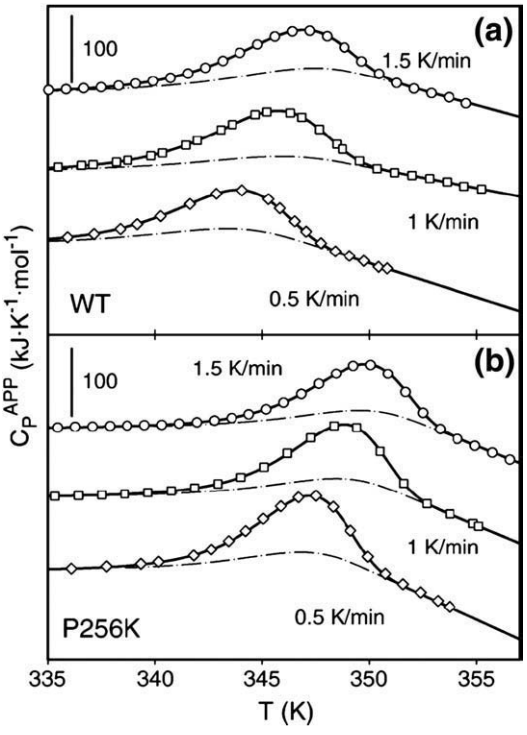

Fig. 1. Scan-rate-dependent scanning calorimetry reveals protein kinetic stability. The calorimetric profiles (heat capacity versus temperature) correspond to the thermal denaturation of the wild-type (a) and P256K (b) variants of lipase from *Thermomyces lanuginosus*. For each variant, several experiments at the indicated scan rates were performed. The strong scan-rate effect reveals that the denaturation process is under kinetic control. The open symbols are the experimental data and the continuous lines are the best fits of the two-state irreversible model (Eq. (6) in Section 2.3).
Figure reprinted from Rodriguez-Larrea et al., J. Mol. Biol. 360 (2006) 715–724 [21] with permission from Elsevier.

human FVIII [95], glutathione s-transferase [99], ceruloplasmin [101], yeast multicopper oxidase Fet3p [105], peroxidase [106], thermophilic phenylalanine hydroxylase [107], the cochlea protein OCP1 [108] and Dr adhesion structures [109].

2.4. General implications of the "success" of the two-state irreversible model

Certainly, for a protein denaturation process that conforms quantitatively to the two-state irreversible model, equilibrium thermodynamics analysis is not valid and no information is available regarding the number and energetic parameters of the protein states significantly populated in a (hypothetical) equilibrium unfolding process [6,12]. Nevertheless, the fact that the *in vitro* thermal denaturation of many proteins conforms to the two-state irreversible model is a central result with important general implications [7]. First, it is clear that the stability of many proteins *in vitro* may have a kinetic basis (i.e., stability is controlled by a rate constant that increases with temperature and the protein denatures in the temperature range in which the denaturation rate becomes significant in the time-scale of the experiment). Secondly, even in these cases of kinetic control of stability *in vitro*, the denaturation temperature determined by DSC is clearly above physiological temperature; therefore, *in vitro* kinetic stability is very likely a reflection of natural selection for *in vivo* kinetic stability [7]. Finally, the possibility of kinetic analyses of thermal denaturation data suggest the possibility of calculating the rate of irreversible denaturation as a function of temperature and subsequently extrapolating to low temperature to obtain estimates of, for instance, the shelf-life of protein pharmaceuticals at 4 °C. The two-state irreversible model itself is possibly too-simple to guarantee the reliability of such a long extrapolation; however, it has contributed to inspire several more sophisticated and promising approaches to the problem of shelf-life prediction from thermal denaturation data [110–114].

3. Early evidence for protein kinetic stability: the α-lytic protease system

The α-lytic protease drew attention in the early nineties as a paradigmatic example of a protein with a folding process under kinetic control [115,116] which could conceivably be an exception to Anfinsen's thermodynamic hypothesis (i.e., the native state might not be at global free-energy minimum). The α-lytic protease (like many other proteases) is synthesized as a proenzyme containing a pro-region which is required for correct folding but which must be subsequently cleaved out to yield the active enzyme. The native, active protease (N) can be unfolded by chemical denaturants and the unfolded state thus obtained (U) can refold back to the active enzyme upon transfer to "native" solvent conditions, but only if a significant amount of the pro-region is added as a separate polypeptide chain. Actually, refolding in the absence of the pro peptide traps the protein in an inactive state with native-like secondary structure (state I) which is stable for weeks, although addition of the pro-region causes its conversion to the native state. Since, both I and N are stable under identical conditions with no detectable conversion (in the absence of added pro polypeptide), it was concluded that the folding of the α-lytic protease is under kinetic and not thermodynamic control [116]. State I readily unfolds to U upon addition of chemical denaturants and its free-energy (in the absence of denaturant) could be estimated as being lower than that of U by 1 kcal or less. States I and N do not equilibrate in any practical time-scale but their relative stabilities could be assessed [8] from the rates of unfolding of N (k_U) and the rate of folding of I (k_F), under the assumption that both processes involve the same transition state. The rate constant k_U could be determined by extrapolation from chemical-denaturant-induced kinetic data, while the measurement of k_F required a high-sensitivity method to estimate the small amount of N formed from I in a laboratory time-scale. Combination of the two rate constants [8] led to the surprising result that, in the absence of the pro-region, the state I, not the N state, is at a minimum free-energy (see Fig. 2). The pro-region plays then a two-fold role: 1) it binds to both I and N, but the binding is stronger in the latter case, thus providing a thermodynamic driving force for folding; 2) It binds to the folding transition state more tightly than to I and, thus decreasing the folding free-energy barrier and providing efficient catalysis of the process. Nevertheless, in the absence of the pro-region, both I and U are more stable than N. Therefore, the stability of the native, active state of the α-lytic protease has a purely kinetic basis and relies on the existence of a high free-energy barrier separating N from other protein states. This situation may be common among the class of pro-dependent proteases, as supported by studies on *Streptomyces griseus* protease B [117], *Nocardiosis alba* protease [118] and *Thermobifida fusca* protease A [119]. Overall, studies on pro-dependent proteases, mainly by Agard and coworkers, have provided a wealth of fundamental information about protein kinetic stability and we will return to these proteins in several sections of this review (6.3, 6.5 and 7.2).

4. How many kinetically-stable proteins are there? Proteomic-scale analysis of kinetic stability

4.1. General evidence in support of widespread kinetic stabilization of proteins

One important question is whether kinetic stability is a widespread feature, introduced during evolution in many protein systems, or rather a property of a small group of proteins which has been naturally-selected as a response to specific functional requirements. We have already provided a partial answer in preceding sections. Irreversible alterations of proteins may easily take place in harsh extracellular conditions and in crowded intracellular environments and, consequently, it appears likely that the biological function of many proteins *in vivo* is linked to a significant degree of kinetic stabilization (Section 1.4). Also, the *in vitro* thermal denaturation of many proteins conforms to the two-state irreversible model and in these cases, the denaturation temperature determined by DSC is

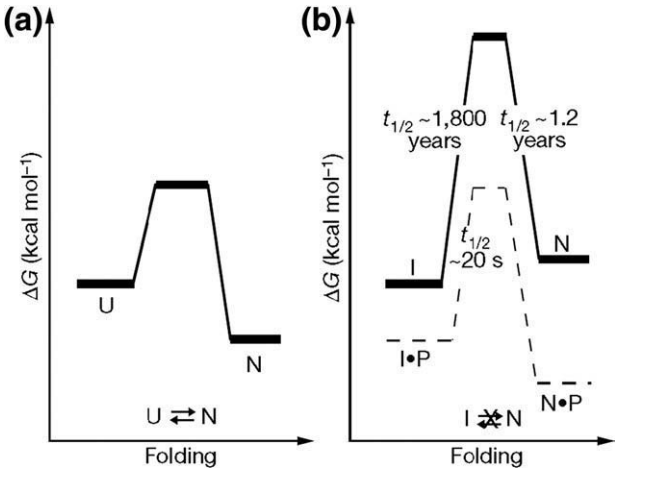

Fig. 2. Kinetic stability in the α-lytic protease system. Panel a (left) depicts a protein that folds to a thermodynamically stable native state through a moderate free-energy barrier. Panel b (right) illustrates the α-lytic protease situation: the native state (N) is thermodynamically unstable with respect to an inactive state (I), and also with respect to the unfolded state (not shown). However, a high free-energy barrier prevents the native state from converting to I in a time-scale of several months. Efficient folding requires a pro-sequence (P), which provides thermodynamic driving force for folding (N·P is thermodynamically stable with respect to I·P) and catalysis (the free-energy barrier is decreased when P is bound: see profile shown by dashed lines).
Figure reprinted from Jaswal et al., Nature 415 (2002) 343–346 [11] by permission from McMillan Publishers Ltd.

clearly above physiological temperature, supporting that the observed *in vitro* kinetic stability is a reflection of natural selection for *in vivo* kinetic stability (Section 2.4). Finally, this conclusion is consistent with the evidence for natural selection of folding/unfolding free-energy barriers that we will discuss in Section 6.2.

The above general arguments do suggest widespread kinetic stabilization of proteins. However, only recently two promising procedures allowing the high-throughput, proteomic-scale analysis of protein kinetic stability have been developed by Colon and coworkers [120] and Marqusee and coworkers [121]. Both approaches are based upon the idea that, since kinetic stability is related to a high free-energy barrier separating the native, biologically-functional protein from the unfolded or partially-unfolded states, it can be probed by a procedure that detects the access of the protein to these non-native states. In the approach of Colon and coworkers, sodium dodecyl sulphate (SDS) is used for this purpose, while the procedure of Marqusee and coworkers relies on the use of proteolytic enzymes. The two methodologies are described below in some detail.

4.2. Determination of kinetic stability on a proteomic scale: the resistance-to-SDS approach

SDS is known to denature proteins. However, unlike common chemical denaturants, its mode of action seems based in its ability to irreversibly trap the proteins during the time in which they are transiently or partially unfolded. Denaturation by SDS seems, therefore, a potential probe of protein kinetic stability; i.e., kinetically-stable proteins are expected to be highly resistant to denaturation by SDS [120,122]. Furthermore, an SDS-resistance assay can be adapted to high-throughput screening of complex mixtures of proteins (cell lysates, for instance). The two-dimensional SDS-PAGE procedure of Colon and coworkers involves the following steps: A) an unheated sample containing the mixture of proteins is subjected to SDS-PAGE; B) the gel lane containing the proteins is cut out, incubated in SDS-PAGE buffer and boiled for 10 min; C) the lane is placed above a larger gel and the second-dimension run is performed. Proteins which are *not* kinetically stable become denatured in step A and, therefore, migrate the same distance in both dimensions of the analysis, thus producing a diagonal line across the gel. On the other hand, kinetically-stable proteins are expected to survive step A (SDS alone) and to denature only in step C (boiling plus SDS): therefore, they will migrate a shorter distance in the first dimension and will appear as below-the-diagonal spots in the gel. Analysis of a cell lysate of *E. coli* using this 2D-SDS approach led to the identification of 50 candidates for kinetically-stable proteins. This number, while significant, is certainly small compared with the number (about 900) of water-soluble proteins that can be detected in traditional 2D gels [123]. It should be noted, however, that kinetic stability is linked to the size of a free-energy barrier and, therefore, is not a yes-or-no property (like pregnancy). Different proteins may show different degrees of kinetic stabilization and we must consider the possibility that the published SDS-resistance studies selected only proteins of very high kinetic stability. A similar case could be made for the proteolysis-based approach described below.

4.3. Determination of kinetic stability on a proteomic scale: the resistance-to-proteolysis approach

Marqusee and coworkers have used proteolytic susceptibility as the basis of a method for energetic profiling of proteins on a proteomic scale [121]. Proteolysis of compact, folded proteins very often implies the transient access to high-energy, partially-unfolded states in which the cleavable states become exposed [121,124,125]. Accordingly, proteolysis may be viewed as a probe of the protein energy landscape, rather than a probe of the folded structure. Marqusee and coworkers [121] subjected an *E. coli* lysate to proteolytic digestions with 0.40 mg/mL trypsin and thermolysin. Surprisingly, multiple proteins survived the proteolytic challenge after four days. A selected number of survivors were identified (22 and 34 from trypsin and thermolysin digestions, respectively) and analyzed in detail. Interestingly, 73% of the trypsin survivors were also thermolysin survivors, supporting that survival is not related to the substrate specificity of the proteases, but it is due instead to some unusual feature of the proteins (or their energy landscapes). An in depth analysis of the maltose binding protein (MBP, one of the survivors common to the trypsin and thermolysin digestions), suggested that, in some cases at least, the unusual feature responsible for survival is the kinetic stability. In fact, the rate of proteolysis by thermolysin of MBP was found to be in good agreement with its rate of global unfolding; this indicates that the free-energy barrier limiting the global unfolding of MBP also determines its kinetic stability in the presence of comparatively high thermolysin concentrations.

4.4. A comparison between the resistance-to-SDS and resistance-to-proteolysis approaches

Clearly, the proteolysis approach of Marqusee and coworkers [121] is meant to probe the essential features of the protein energy landscape on a proteomic scale. Still, it appears reasonable to assume that a significant number of the survivors from the four-day proteolysis challenge must be endowed with high kinetic stability. It is, therefore, rather puzzling that only 6 proteins overlap in the proteolysis study of Marqusee and coworkers and the SDS-analysis of Colon and coworkers described above [120]. Reasons for this difference have been discussed [120]. One interesting possibility (although speculative at this stage) would be that both procedures are sensitive to kinetic stability, but identify different types of kinetically-stable proteins as they trap/probe different types of partially-unfolded states (states with large exposure of hydrophobic residues in the case of SDS and states with exposure of the protein backbone in the case of proteolysis). There is, nevertheless, one important point in which both analyses agree [120,121]: most of the proteins identified have had their structures solved by X-ray crystallography (or are linked to close homologues of known structure). Again, an interesting possibility is that kinetically-stable proteins are advantageous for X-ray crystallography, due possibly to enhanced resistance to degradation or aggregation and ease of purification or crystallization.

5. Kinetic stability and misfolding diseases

5.1. Introductory remarks

A variety of human diseases are known to be associated with protein misfolding processes leading, for instance, to accelerated protein degradation, improper trafficking or protein aggregation with formation of amyloid fibers. The role of destabilizing environments and/or mutations in these processes has long been recognized [126–128]. "Generalized" Lumry–Eyring models [7],

$$\textit{Native state} \leftrightarrow \{\textit{Non-native ensemble}\} \rightarrow \textit{Irreversibly denatured protein} \quad (7)$$

do suggest a role of, specifically, kinetic stability in misfolding. Amyloid formation, for instance, could be viewed as an irreversible denaturation process that occurs through an amyloidogenic intermediate (a member of the non-native ensemble), the accessibility of which (in a biologically-relevant time-scale) likely depending on the kinetic stability of the native protein (i.e., on the free-energy barrier "separating" the native state from the non-native ensemble). In addition, as we discuss in Section 5.2, kinetic stability is very sensitive to mutational effects, which further supports its involvement in misfolding diseases. Finally, but most important, a number of

experimental studies on several diseases point to the existence of a direct link between protein misfolding *in vivo* and deleterious effects on kinetic stability. Some of these experimental studies are summarized in Sections 5.3, 5.4 and 5.5.

5.2. High-sensitivity of kinetic stability to mutational effects suggests its involvement in misfolding diseases

Kinetic stability is related to the time-scale in which a protein can remain in the native functional when its stability is determined by the "crossing" of a free-energy barrier. According to transition-state theory, this time-scale is $\tau = 1/k$ with k given by Eq. (4). Accordingly,

$$\tau \propto \exp\left(\frac{\Delta G^{\neq}}{RT}\right) \tag{8}$$

and the time-scale changes in an exponential manner with the activation free-energy value. As a result, even a decrease of a few kJ/mol in $\Delta G^{\neq}$ can produce a significant change in τ [129], from a value, for instance, of about several weeks to a value on the order of hours or even minutes (Fig. 3). The situation is dramatically different in the case of mutational effects on thermodynamic stability, at least if these are measured by the equilibrium denaturation temperature (the temperature, T_m, at which half of the protein molecules are unfolded under equilibrium conditions and the equilibrium constant for unfolding – Eq. (2) – equals unity). T_m values can easily be shown (Fig. 3) to shift typically only a few degrees upon a change in unfolding free-energy (Eq. (3)) of a few kJ/mol. A T_m shift of a few degrees may be expected to be of little consequence by itself if the T_m values are well above physiological temperature [129].

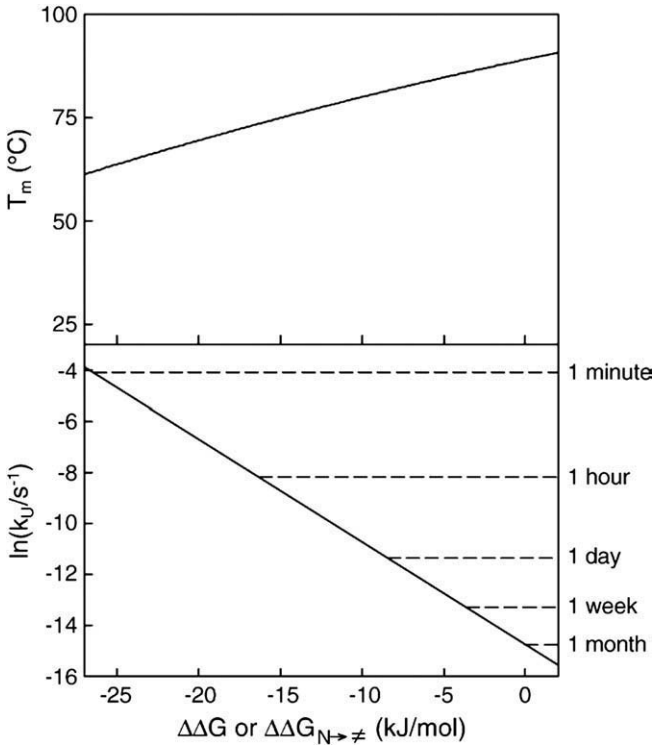

Fig. 3. Mutational effects on thermodynamic stability versus mutational effects on kinetic stability. The upper panel shows the dependence of the equilibrium denaturation temperature with the mutational effect on the unfolding free-energy change ($\Delta\Delta G$). The T_m values are calculated by solving numerically the Gibbs–Helmholtz equation using the unfolding thermodynamic parameters of thioredoxin (for details, see [129]). The lower panel shows the dependence of the thioredoxin unfolding rate constant with the mutational effect on activation free energy ($\Delta\Delta G_{N\rightarrow\neq}$). Rate constants are calculated using transition-state theory (for details, see [129]). Representative half-life times are shown in the right axis. Note that a decrease of a few k/mol in activation free energy can produce a large change in the unfolding time-scale. Figure reprinted from Godoy-Ruiz et al., J. Mol. Biol. 362 (2006) 966–978 [129] with permission from Elsevier.

The above general ideas may provide one clue as to why misfolding diseases are in some cases linked to conservative mutations (as an illustrative example, human triose phosphate isomerase deficiency, an autosomal disease that causes premature death, is very often caused by a very conservative E104D mutation in the triose phosphate isomerase gene [130]). Protein variants with disruptive mutations may be highly prone to misfolding, but the quality control systems of the cell [131,132] are likely to eliminate them. Variants with conservative mutations, on the other hand, may escape the quality control systems but still show a decreased kinetic stability that allows misfolding to occur in a biologically-relevant time-scale.

5.3. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease (after the New York Yankees baseball player who died from ALS in 1941), is a motor neurone syndrome that affects both upper and lower motor neurons [133], causing death from respiratory failure, usually within a few years from diagnosis (a prominent exception being physicist Stephen Hawking). Its causes remain unclear in most cases. However, inherited familial ALS (FALS) accounts for about 10% of ALS occurrences and mutations (more than 100) of the superoxide scavenger Cu/Zn superoxide dismutase (SOD) have been associated with about 20% of the cases of FALS [134,135]. The disease does not appear to be caused by low SOD activity levels and, in fact, mice in which SOD is completely deleted do not show motor neuron disease [133,136]. On the other hand, impaired incorporation of Cu in mice lacking the copper chaperone for SOD [137] does not prevent the ALS-associated mutations from provoking the disease, suggesting that the metal-deficient (apo) precursor state of SOD causes neurotoxicity. Furthermore, Oliveberg et al. [135] found that ALS-mutations significantly decrease the stability of apo-SOD with the destabilizing effect showing a correlation with the mean survival time of patients. A number of more recent studies [138–142] further support the essential role of metals in the folding free-energy landscape of SOD and that metal removal is likely linked to misfolding. A plausible scenario is that the pathogenic mechanism of ALS involves aggregation of apo-SOD species of reduced stability. The possibility that reduced kinetic stability does play a key role in ALS is clearly suggested by the studies of Colon and coworkers [143,144], who found that the absence of metals kinetically destabilize wt and mutant SOD leading to a 60-fold increase in the rate of unfolding, and also by those of Meiering and coworkers [145] who found that ALS-associated mutations decrease the stability of holoSOD mainly by increasing unfolding rates, and favour the formation of Zn-free monomeric intermediates.

5.4. Phenylketoenuria

Phenylketoenuria (PKU) is a loss-of-function metabolic disease caused by misfolding of mutated phenylalanine hydroxylase (PAH: a cytosolic enzyme involved in hepatic catabolism of L-phenylalanine) with the subsequent accumulation of L-phenylalanine (L-Phe) in blood and brain. If the condition is left untreated, it may lead to problems in brain development and mental retardation. The disease is currently treated by an L-Phe-restricted diet, but this diet therapy is expensive, socially-burdening and needs to be maintained "for life"; thus alternative treatments are being actively sought [146]. Regarding the number of patients, PKU is on the verge of being a rare disease; however, with more than 500 mutations identified in the PAH gene

[www.pahdb.mcgill.ca/] and a wide variability in the phenotype of patients, PKU is becoming a model disease for the development of patient-tailored treatments [146]. PAH requires (6R)-L-*erythro*-5,6,7,8-tetrahydrobioterin (BH_4) as an essential cofactor and recent work [147,148] has shown that oral administration of the cofactor BH_4 leads to normalization of L-phenylalanine levels and may be an effective therapy for PKU patients with certain PAH mutations (a listing of BH_4-responsive genotypes can be found at www.bh4.org/BH4DatabasesBiopku.asp). In some of these cases, BH_4 appears to stabilize the enzyme with subsequent protection from inactivation and proteolytic degradation [147]. Furthermore, Martinez and co-workers [146] have shown that increasing concentrations of BH_4 decrease the rate of irreversible denaturation of wt and mutant forms of PAH, supporting that the therapeutic action of BH_4 may be linked to its efficiency at compensating mutation-induced kinetic destabilization of the native state of PAH. From a more general viewpoint, the studies on PKU-correction by BH_4 have pointed to the possibility of finding alternatives to the treatment of PKU through the screening of chemical libraries in search of substances that stabilize and promote the correct folding of PAH [149]. It appears plausible that the "pharmacological chaperones" thus found will act partly as kinetic stabilizers of the native, functional state.

5.5. Transthyretin (TTR) amyloidoses

5.5.1. General features of TTR-amyloidoses

The two examples discussed above (amyotrophic lateral sclerosis and phenylketoenuria) suggest a relation between protein misfolding diseases and kinetic stability. This relation, however, has been probed in detail in the case of transthyretin amyloidoses, due mostly to the studies of Kelly and coworkers over many years. Transthyretin (TTR) is a soluble protein that is secreted into the blood and cerebrospinal fluid. Its functional native state is a tetramer that transports thyroxine (T_4) and the retinol binding protein with retinol bound (holoRBP). TTR is related to a number of amyloid diseases [150]: senile systemic amyloidosis (SSA), familial amyloid polyneuropathy (FAP), familial amyloid cardiopathy (FAC) and familial central-nervous-system amylodosis. SSA involves deposition of wt-TTR and is a common age-related disorder, with about 25% of people over 80 having amyloid deposits in the heart. The familial TTR-amyloidoses are associated with the deposition of TTR variants, with several of them showing tissue-selective deposition and pathology. It has been noted [150] that, while familial TTR-amyloidoses have been traditionally considered as rare diseases, recent improvements in diagnosis reveal a large number of patients worldwide. Currently, the only effective treatment for the familial TTR-amyloidoses is liver transplantation, first performed in 1990 [151].

5.5.2. Role of kinetic stability in TTR-amyloidoses

Early biophysical studies [152–154] ruled out the native tetrameric TTR as the amyloidogenic precursor, established that partial denaturation of TTR into a monomeric state of altered structure is required for amyloid fibril formation *in vitro* and suggested, therefore, the stabilization of the native state as a promising approach to inhibit fibril formation. It soon became evident, however, that the effect of TTR-amyloidoses mutations on thermodynamic stability alone could not reliably predict the severity of the disease and that the rate of native tetramer dissociation (required for amyloid fibril formation) played a key role [155]. A relevant illustration is afforded by the V122I variant of TTR, responsible for most cases familial amyloidotic cardiomyopathy (FAC), primarily in individuals of African descent. While tetramer dissociation in wt-TTR is quite slow, the V121I mutation significantly decreases the initial free-energy barrier for native-state denaturation, leading to a several-fold enhancement in the rate of tetramer dissociation [156]. The interplay between the effects of the mutations V30M and T119M provides another particularly clear example. V30M is the most frequent cause of familial amyloid polyneuropathy (FAP) in heterozygotes, but the presence of the T119M mutation on the second TTR allele ameliorates the FAP disease in V30M carriers [157]. This "trans-suppression" effect appears to have mainly a kinetic basis [158], since V30M/T199M hybrid tetramers show a decrease in the rate tetramer dissociation (with the concomitant decrease in the rate fibril formation) as the number of subunits with the T199M mutation is increased.

5.5.3. Kinetic stabilizers of native TTR may be efficient inhibitors of fibril formation and may provide alternatives to liver transplantation in the treatment of TTR-amyloidoses

The fact that the trans-suppressor mutation T199M acts mainly through kinetic stabilization (see above) immediately suggested [158] that substances with the capability to increase the free-energy barrier for the rate-limiting dissociation may be efficient inhibitors of fibril formation (Fig. 4). In this context, small molecules that bind to the thyroid hormone (thyroxine: T_4) sites provide a workable approach [154], since T_4 binding globulin is the major T_4 carrier in plasma, with TTR being a secondary carrier (only a small fraction of the plasma TTR has T_4 bound). A number of such small-molecule inhibitors of TTR amyloid formation have been reported in recent literature [159–167]. Two of these substances are discussed is some detail below.

Genistein is the major isoflavone natural product in soy. It is considered a "nutraceutical" (a substance with both nutritional value and medicinal benefits) and has been evaluated for the treatment of several diseases [162]. Genistein has been reported to be an excellent inhibitor of TTR tetramer dissociation and fibril formation [162]. It works as a kinetic stabilizer by binding to the hormone binding sites in TTR and raising the free-energy barrier for native tetramer dissociation. Furthermore, genistein appears to show highly selective binding to TTR over all the other plasma proteins. These results suggest the interesting possibility that some patients of TTR-amyloidoses could benefit from increasing their intake of soy products [162].

Recently, tafamidis meglumine (known as FX-1006A) has been developed as TTR kinetic stabilizer to treat TTR-amyloidoses. A Phase I study in healthy volunteers showed FX-1006A to be safe and to have strong TTR stabilization in the plasma of participants [150]. The results of the Phase II/III trials were released in July-2009. As described in "Nature Biotechnology" [167], the studies involved 128 patients of FAP carrying the V30M mutation in TTR and, over the 18-month study, oral administration of FX-1006A was found to stop the progression of the disease, while patients on placebo worsened. These results support that kinetic stabilizers of native tetrameric TTR may provide a promising alternative to liver transplantation in the treatment of TTR-amyloidoses.

6. Natural selection for protein kinetic stability

6.1. Protein evolution and protein stability

Recent work [129,168–175] has emphasized the relation between protein stability and protein evolution. A simple argument is that most mutations in a protein affect stability and not function and, furthermore, that most mutations are expected to be destabilizing; accordingly, accumulation of a few mutations can compromise "protein fitness" [175,176] due to the concomitant sharp decrease in protein stability. In addition, the destabilizing effects of mutations is often assumed to limit the acquisition of new activities, as stability and the new function are expected to trade-off (as an often-cited example, some mutations conferring TEM-1-lactamase with resistance against third-generation antibiotics were found to be destabilizing [177]). Most of these studies appear to discuss the relation stability-evolution in terms of thermodynamic stability, perhaps partly as a result of the availability of several computational algorithms to estimate mutation effects on unfolding free-energy [178]. Nevertheless, the relevance of

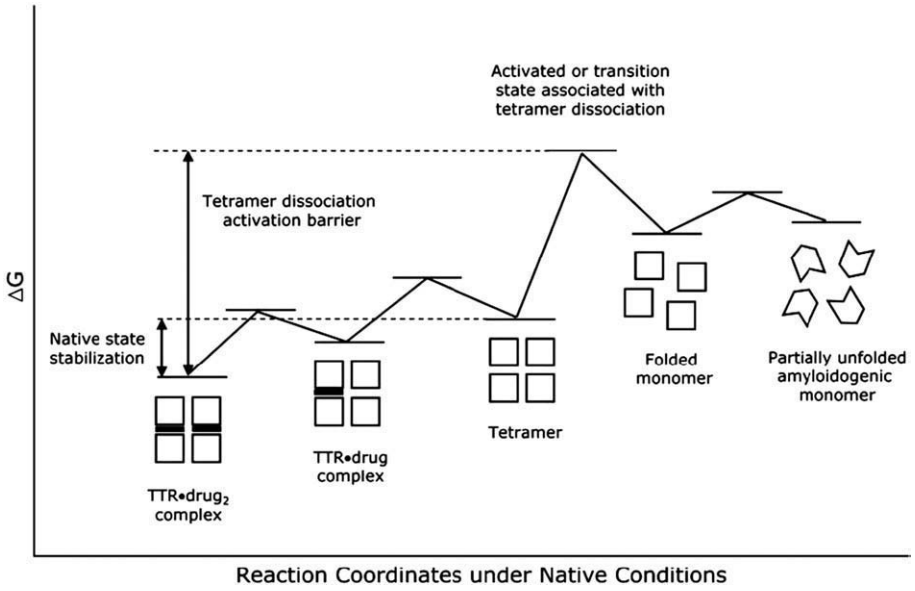

Fig. 4. Free-energy profile illustrating the kinetic stabilization of native, tetrameric transthyretin with concomitant decrease in the rate of amyloid fibril formation. Note that ligand binding to the native protein increases the activation free-energy barrier that determines the rate of the overall process.
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kinetic stability for protein molecular evolution is specifically recognized in several works [129,175] and, as discussed below, clear evidence for natural selection of protein kinetic stability can be found in the published literature.

6.2. Natural selection of folding/unfolding free-energy barriers

A particular clear, albeit indirect, evidence for natural selection of kinetic stability stems from the realization in recent years that the significant free-energy barriers often observed in protein folding–unfolding processes are not a requirement of protein (heteropolymer) physics. After all, folding–unfolding processes involve only the formation and break-up of weak, non-covalent interactions and, therefore, high free-energy barriers, as in chemical reactions involving covalent bonds, are not to be expected. In fact, studies on small fast-folding proteins have revealed marginal free-energy barriers [48,51,179–181] and, in some cases, have provided experimental evidence [47,49,50,182–188] for the existence of barrierless protein folding, i.e., the downhill folding scenario of Wolynes and Onuchic [189]. In addition, studies on de novo designed proteins (i.e., proteins lacking an evolutionary history or "poorly-evolved" proteins) point to a number or revealing features [52,190–192], such as rugged folding landscapes, complex conformational dynamics, low folding cooperativity and, most significantly, rapid folding even in the absence of explicit design or selection for low free-energy barriers and fast kinetics. All these studies support that the significant barriers found in many kinetic studies on the folding–unfolding of natural proteins are not a fundamental physical requirement but a product of natural selection. Actually, natural selection for kinetic stability seems to provide a straightforward evolutionary rationale for these barriers. First of all, partially-unfolded protein conformations (or microstates) are prone to undergo undesirable aggregation processes and a significant free-energy barrier "separating" the native and unfolded macrostates (or ensembles) of a protein actually means that these partially-unfolded conformations have high free-energy and are not energetically favoured [47,181,193]. Secondly, in a Lumry–Eyring scenario, a significant unfolding barrier kinetically protects the native protein against a variety of irreversible alteration processes that may occur through unfolded or partially-unfolded macrostates [7], as we have discussed in Section 1.

6.3. Natural selection for kinetic stability may be suggested by atypical behaviour associated to the kinetics of unfolding or irreversible denaturation

Indirect, but compelling evidence supporting natural selection for kinetic stability is sometimes revealed by the "anomalous" or "atypical" behaviour associated to the kinetics of unfolding or irreversible denaturation. For instance, kinetically-stable proteases (such as the α-lytic protease: Section 3) have been reported to simultaneously show an anomalously large activation heat capacity changes for unfolding and high unfolding activation free-energy, which is maximum at the optimal growth temperature for the organism [194]. Another example is provided by a recent comparative analysis [4] into the irreversible thermal denaturation of triosephosphate isomerases (TIMs) from three different organisms: *Trypanosoma cruzi*, *Trypanosoma brucei* and *Leishmania mexicana*. The study revealed surprising differences between the kinetic denaturation parameters for these three proteins, with *T. cruzi* TIM showing a much larger activation energy value and, consequently, a much lower room temperature, extrapolated denaturation rates. These results support that proteins sharing the same structure and function but belonging to different organisms may have been naturally-selected to have widely different kinetic stabilities, even if the relation of this "between-species" variation in TIM kinetic stability with the biological fitness of the three organisms (*T. cruzi*, *T. brucei*, *L. mexicana*) may not be currently clear [4].

6.4. Natural selection for kinetic stability is a likely origin of correlations between mutational effects on protein energetics and sequence-alignment residue-statistics

Recent years have witnessed an exponential increase in the size of sequence databases. A simple database search using as query the sequence of one's favourite protein is likely to yield a large of number of sequences, corresponding to proteins sharing the same function and/or fold with the query and belonging in most cases to different organisms. The sequences resulting from the search and the query sequence can then be aligned using a variety of algorithms. Surprisingly, a number of studies [129,168,169,195–199] have reported significant correlations between mutations effects on protein stability and frequencies of occurrence of residues in sequence-alignments. The simplest evolutionary model that can explain such a correlation involves an

evolutionary threshold for stability [129,168,169,175] in such a way that mutations that bring protein stability below the threshold compromise organism fitness and are rejected during evolution (purifying natural selection), while mutations that keep stability above the threshold are neutral and may become fixed. In this scenario, a mutation is rejected or fixed depending on the previous mutational background (which determines the previous stability level) and, if fact, mutation fixation becomes a stochastic-like process with probabilities related to the mutation effect on stability, thus rationalizing the observed correlations with sequence-alignment. An important question in this context is whether the evolutionary threshold is associated to thermodynamic stability or to kinetic stability. This issue has been recently addressed by Godoy-Ruiz et al. [129] using *E. coli* thioredoxin as a model system. These authors studied 27 variants of thioredoxin each containing a single, conservative mutation (involving valine ↔ isoleucine or glutamate ↔ aspartate substitutions) and found a significant correlation between mutation effects on stability and sequence-alignment statistics. Then, they derived an estimate of the evolutionary stability threshold for thioredoxin from a Monte Carlo simulation of the natural-purifying-selection/neutral-evolution scenario constrained by the parameters of the experimental stability/sequence-statistics correlation. The striking result was that, in thermodynamic terms, the threshold level is only a few kJ/mol below the thermodynamic stability of wt thioredoxin from *E. coli*. As elaborated in Section 5.2 (see Fig. 3), a change of few kJ/mol in unfolding free-energy translates into a moderate effect on equilibrium denaturation temperature, while a similar change in activation free-energy may significantly affect the unfolding half-life and, consequently, the time-scale for irreversible denaturation processes. Clearly, an evolutionary threshold of a few kJ/mol below wt stability is suggestive of natural selection for kinetic stability. This interpretation was supported [129] by experimental studies on the rates of unfolding of the 27 thioredoxin variants which demonstrated a significant correlation between mutation effects on the free-energy barrier for unfolding and sequence-alignment statistics. Overall, these results suggest that purifying natural selection may often operate on the basis of kinetic stability thresholds (associated, for instance, to the half-life of the functional protein *in vivo*).

6.5. The relation between kinetic stability, natural selection and biological function

Natural selection for kinetic stability is apparent in those cases in which the link with biological function can be readily established. The structural homologues α-lactalbumin (BLA) and hen-egg-white lysozyme (HEWL) provide a striking example. These two proteins have 38% sequence identity and share the same overall tertiary structure and folding topology. Despite this, their thermodynamic folding–unfolding barriers (as determined by a variable barrier-analysis of scanning calorimetry thermograms: [47]) are dramatically different, with BLA showing a marginal barrier (on the order of the thermal energy) and HEWL having a significant barrier of about 40 kJ/mol or higher [181]. This difference, however, can be rationalized [181] in terms of natural selection in response to specific biological requirements. BLA binds to membranes by adopting partially-unfolded conformations (usually described as a "molten globule": [200,201]) which are favoured by a marginal folding–unfolding barrier. On the other hand, HEWL is a bactericide that must work under very harsh extracellular conditions (a Lumry–Eyring scenario: see Section 1) and a high unfolding free-energy barrier will increase its kinetic stability (i.e., its half-life in the active form) thus producing a more efficient bactericide.

Secreted proteases, such the α-lytic protease (Section 3), provide another arresting example of the link between natural selection, kinetic stability and biological function. Their biological function is to provide nutrients for the bacterium and, since they work in the soil, they are naturally-selected for survival under very harsh, highly proteolytic conditions, which implies that they must be endowed with high kinetic stability. In the case of the α-lytic protease the high kinetic stability has been found to be associated [11], not only to a high free-energy barrier for the global denaturation process but, also, to the elimination of transient, local-unfolding processes with the concomitant enhancement in resistance to proteolysis. As discussed in Section 3, the native state of the α-lytic protease is thermodynamically unstable, its folding being made possible by a pro-region which provides catalysis and a thermodynamic driving force for the process and which is cleaved out upon completion of folding. The interesting proposal has been made [11] that the removal in the α-lytic protease of the constraints associated to thermodynamic stability allows the evolution of special properties, such as the restricted native-state dynamics and the associated suppression of proteolytic sensitivity. The general implication is that kinetic stability may provide a mechanism for the evolution of optimal functional properties [11,118].

7. The molecular basis of protein kinetic stability

7.1. Is there a relation between protein fold/structure and kinetic stability?

Possibly the most basic question regarding the molecular basis of kinetic stability is whether some specific protein structures or folds are required for kinetic stability or are more prone to lead to kinetic stabilization. Although a definitive answer does not seem possible at this stage, the currently available experimental evidence does not appear to reveal a clear relation between kinetic stability and protein structure/fold. For instance, the studies of Marqusee and coworkers [121] (see Section 4.3) did not highlight any common structural features that could explain proteolytic resistance. Likewise, the studies of Colon and coworkers on resistance to denaturation by SDS [120] (see Section 4.2) do not show any dramatic trends, except perhaps for a lower percentage of SDS-resistant proteins among those with all α-helical structure and a higher percentage among those with the higher degrees of oligomerization. It was hypothesized that monomeric predominantly helical proteins cannot easily attain the level of topological complexity required for kinetic stability [120].

7.2. Kinetic stabilization through the break-up of interactions in the relevant transition state

Overall, the experimental studies described in the preceding section support the notion that natural selection can endow many different protein structures with kinetic stability by using the variety of strategies available in the structural-energetics toolbox. According to this view, an understanding of the molecular basis of kinetic stability is afforded by a knowledge of the molecular interactions that contribute to the relevant free-energy barriers or, equivalently, by an experimentally-supported structural model of the corresponding transition state. The studies of Kelch and Agard [119] on the structural mechanism of the kinetic stability of pro-dependent proteases (the class to which the α-lytic protease – see Section 3 – belongs) provide an excellent example. These proteins share with all the members of the chymotrypsin fold a canonical double β-barrel structure with the active state including residues from the N- and C-terminal domains. Unlike the typical chymotrypsin fold, however, their N- and C-terminal domains are connected by a β-hairpin known as the "domain bridge" (see Fig. 5). Structural determinations revealed striking disparities in domain bridge architecture between different members of the pro-dependent protease class. Furthermore, Kelch and Agard [119] analyzed four pro-dependent proteases (α-lytic protease, *S. griseus* protease B, *N. alba* protease, and *T. fusca* protease A) and found a highly significant correlation between the unfolding free-energy barrier and the surface area (either total or hydrophobic) buried upon domain bridge folding and docking to the N- and C-terminal domains. The implication (supported by studies on chimeric proteases) is that

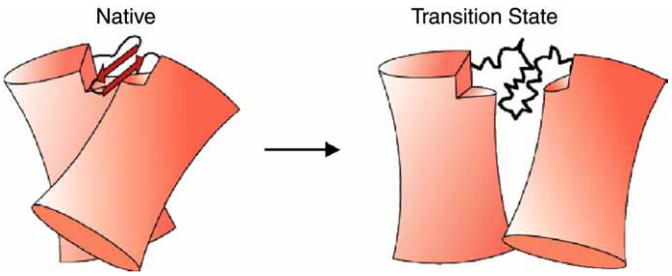

Fig. 5. Model for the unfolding transition state of pro-dependent proteases. In the transition state (right) the domain bridge partially unfolds and breaks many contacts between the C- and N-terminal domains. This break-up of interactions in the transition state (as compared with the native state: left) contributes to an enhanced free-energy barrier and a high kinetic stability.
Reprinted from Kelch and Agard, J. Mol. Biol. 370 (2007) 784–795 [119] with permission from Elsevier.

the domain bridge modulates the kinetic stability through its packing interactions with the rest of the protein. The idea is adequately summarized by the transition-state model shown in Fig. 5 in which the domain bridge becomes partially unstructured with concomitant undocking of the three domains (bridge, C-terminal and N-terminal). This implies that a large number of interactions are broken in the transition state (as compared with the native structure: see Fig. 5) thus contributing to a high free-energy barrier and an enhanced kinetic stability.

Another revealing example is afforded by the role of electrostatic (charge–charge) interactions [181] on the widely different thermodynamic unfolding barriers of the structural homologues α-lactalbumin (BLA) and hen-egg-white lysozyme (HEWL) (see Section 6.5). Most charged residues in proteins are found at the surface involved in favourable interactions with the aqueous solvent. Although these "hydrated" residues are expected to exert comparatively weak Coulomb effects, the sum of many such small effects may have relevant consequences and, in fact, the design of the surface-charge distribution has been shown to be an efficient modulate protein properties [202–212]. In the case of BLA and HEWL, both proteins share the same overall tertiary structure but have very different surface-charge distributions. Halskau et al. [181] used an Ising-like statistical-model [213] to generate computationally all possibly partially-unfolded conformations of BLA and HEWL according to the double-sequence approximation. The electrostatic analysis of the resulting ~10^7 microstates showed that, in the case of BLA, the interactions between surface charges stabilize partially-unfolded conformations (with respect to the native state) while, in the case of HEWL, they preferentially stabilize the native state. The implication [181] is that favourable electrostatic interactions present in native HEWL are likely broken in the transition state for unfolding, thus contributing to a high free-energy barrier and to the kinetic stability required for HEWL to work as a bactericide under harsh extracellular conditions (see Section 6.5).

Finally, the notion that the break-up of stabilizing interactions in the kinetically-relevant transition state contributes to kinetic stability immediately suggests approaches for stability enhancement of proteins of biotechnological interest, as illustrated by recent work on lipase from *Thermomyces lanuginosus* [21]. Heated molecular dynamics simulations were used to determine regions of "high-flexibility" in lipase molecule which are likely to be disrupted in the transition state for lipase irreversible denaturation. In fact, a directed-evolution approach focused to these "weak" regions led to several lipase variants with highly-enhanced kinetic stability [21].

7.3. Non-native energetic balance in transition states: the role of solvation barriers in protein kinetic stability

The examples given in the preceding section seem to imply a picture of the transition state for protein unfolding (or irreversible denaturation) in terms of regions that remain essentially native-like, while parts of the molecule unfold (or "undock") with a concomitant break-up of interactions that contribute to the free-barrier and, ultimately, to kinetic stability. This view, however, may be over-simplistic in some cases, in particular if the transition states show a non-native energetic balance. Early evidence supporting a non-native energetic balance in transition states for protein unfolding can be found in pioneering work from C.R. Matthews lab [214]. These authors carried out a systematic study of the urea and temperature dependences of the unfolding kinetics for the α-subunit of tryptophan synthase. They reported energetic evidence for a low exposure to the solvent in the transition state and, at the same time, they determined a significant value for the activation energy of the unfolding process (about 100 kJ/mol at 25 °C in the absence of urea). These results do not appear compatible with a simple view of the transition state as a partially-unfolded native structure and suggest instead the relevance of non-native structural energetics. More recent work [4,21] has provided further support for a non-native energetic balance in transition states for unfolding and irreversible denaturation processes and, as elaborated below, has identified the so-called "solvation barriers" (associated to the finite size of water molecules) as a likely non-native contribution to transition-state energetics.

A number of theoretical and computational studies have suggested the existence of desolvation/solvation barriers in protein folding/unfolding processes [215–220]. In simple terms, a solvation barrier (in an unfolding or irreversible denaturation process) may be viewed as a result of the asynchrony between water penetration and the break-up of internal interactions. In other words, protein segments must separate before water molecules can penetrate and establish close contact with the protein surface and, accordingly, the transition state for unfolding (or irreversible denaturation) will be characterized by a network of water-unsatisfied, partially-broken internal contacts (see Fig. 6 for a pictorial illustration). This network will make a large contribution to activation energetics which is not coupled to exposure to solvent. As recently proposed [4,21,221], therefore, solvation-barrier contributions to protein kinetic processes can be experimentally assessed by confronting experimental activation energies with the values of adequate measures of the exposure to solvent in the transition state (such as, for instance the activation $m^{\ddagger}$ values derived from the urea-dependence of the unfolding kinetics). Very recently,

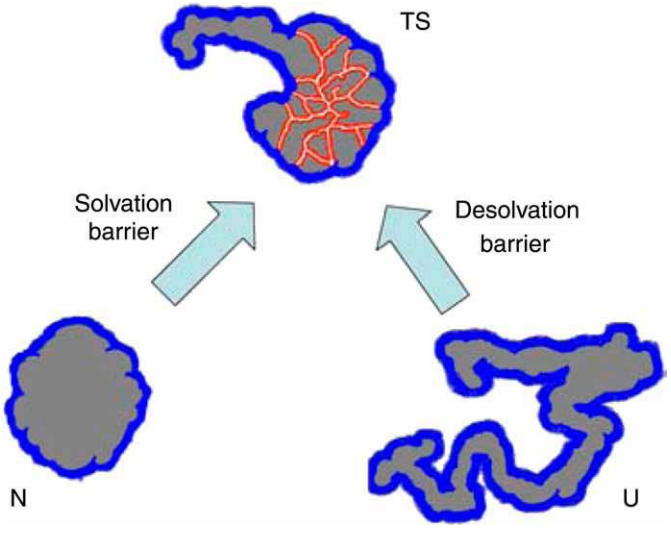

Fig. 6. Pictorial illustration of solvation/desolvation barrier contributions to protein folding and denaturation processes. The surface shown in blue in the native (N), transition state (TS) and unfolded state (U) is exposed to the solvent. The surface shown in red in the transition state represents broken internal contacts which are not solvated by water.
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this approach has been applied to the irreversible denaturation of two protein systems: lipase from *T. lanuginosus* [21] and triosephosphate isomerases from several Trypanosomatidae [4]. In both cases, solvation-barrier effects were found to be main determinants of kinetic stability. Most importantly, however, large differences in solvation-barrier contributions were found for triosephosphate isomerases from different species implying that kinetic solvation effects provide an efficient mechanism for the modulation of free-energy barriers within a given protein scaffold [4]. The structural and evolutionary details of this mechanism remain, however, to be elucidated.

Note added in proof

A review paper relevant to section 5.5 has been recently published online: S. Connelly, S. Choi, S.M. Johnson, J.W. Kelly, I.A. Wilson, Structure-based design of kinetic stabilizers that ameliorate the transthyretin amyloidoses, Curr. Opin. Struct. Biol. (2010), doi:10.1016/j.sbi.2009.12.009.

Acknowledgments

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