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Review

Review

Kinetic trapping in protein folding

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Abstract

The founding principles of protein folding introduced by Christian Anfinsen, together with the numerous mechanistic investigations that followed, assume that protein folding is a thermodynamically controlled process. On the other hand, this review underscores the fact that thermodynamic control is far from being the norm in protein folding, as long as one considers an extended chemical-potential landscape encompassing aggregates, in addition to native, unfolded and intermediate states. Here, we highlight the key role of kinetic trapping of the protein native state relative to unfolded, intermediate and, most importantly, aggregated states. We propose that kinetic trapping serves an important role in biology by protecting the bioactive states of a large number of proteins from deleterious aggregation. In the event that undesired aggregates were somehow formed, specialized intracellular disaggregation machines have evolved to convert any aberrant populations back to the native state, thus restoring a fully bioactive and aggregation-protected protein cohort.

Key words: kinetic trapping, kinetics, metastability, protein folding, thermodynamics

General aspects of protein folding

Understanding how proteins achieve their three-dimensional structure (i.e. how they fold) is one of the most compelling problems in modern biology. While researchers made considerable progress elucidating the folding mechanism of purified proteins *in vitro* (Dobson, 2003; Englander *et al.*, 2007; Fersht, 2008; Schaeffer *et al.*, 2008; Barrick, 2009; Thirumalai *et al.*, 2010; Bavishi and Hatzakis, 2014; Gelman and Gruebele, 2014), the folding mechanisms in complex cell-like environments are still poorly understood (Gething and Sambrook, 1992; Fedyukina and Cavagnero, 2011; O'Brien *et al.*, 2011; Kim *et al.*, 2015; Rodnina, 2016; Javed *et al.*, 2017). Most importantly for this review, the relation and potential interplay between protein folding and aggregation, in both *in vitro* and cell-like environments, has only begun to be explored at a quantitative level (Chiti *et al.*, 2002; Dobson, 2004; Chow *et al.*, 2006; Naeem and Fazili, 2011; Neudecker *et al.*, 2012).

In 1969, Cyrus Levinthal theorized that unfolded proteins refold via specific pathways (as opposed to via random routes), given that the experimentally observed folding time limits the number of conformations that a protein is able to sample (Fig. 1A) (Levinthal, 1969).

In 1973, following his pioneering studies on ribonuclease A (Anfinsen *et al.*, 1961), Christian Anfinsen formulated the well-known thermodynamic hypothesis, which states that the folding of any given protein leads to formation of the most thermodynamically stable conformation (Fig. 1B) (Anfinsen, 1973). This hypothesis applies within any given environment, defined as a combination of buffer conditions, pH, temperature, pressure, target-protein concentration and concentration of any other pertinent components (Anfinsen, 1973).

Specifically, as schematically illustrated in Fig. 2, Anfinsen showed that ribonuclease A folds under thermodynamic control, given that its urea-denatured reduced state can reversibly turn into the native bioactive conformation after dilution into buffer in the presence of traces of 2-mercaptoethanol. This important experiment showed that proteins can adopt their native state based only on primary structure and environment (Anfinsen, 1973). Anfinsen's experiments also showed that several small single-domain monomeric proteins fold and unfold fast and reversibly, consistent with the thermodynamic hypothesis (Epstein *et al.*, 1963; Jackson, 1998).

According to Levinthal's (Fig. 1A) and Anfinsen's (Fig. 1B) predicaments, and assuming that the unsampled conformations proposed

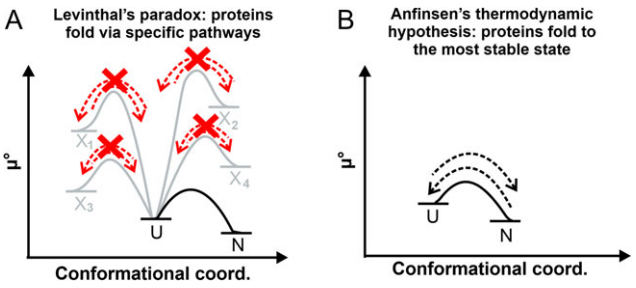

Fig. 1 Protein folding is governed by fundamental principles. (A) Refolding-time arguments limit the number of available folding pathways. Therefore, consistent with Levinthal's paradox (Levinthal, 1969), proteins attain their native state by traveling through folding routes characterized by thermally accessible chemical potential barriers. Red dashed lines with a red cross highlight the lack of kinetic accessibility of the gray routes. U and N denote unfolded and native states, respectively. X_n denotes alternate inaccessible protein conformational states. **(B)** The native state is the most stable conformation of a protein under physiologically relevant conditions. Native, unfolded and intermediate states interconvert freely, under these conditions. *U* and *N* denote unfolded and native states, respectively (Jackson, 1998)

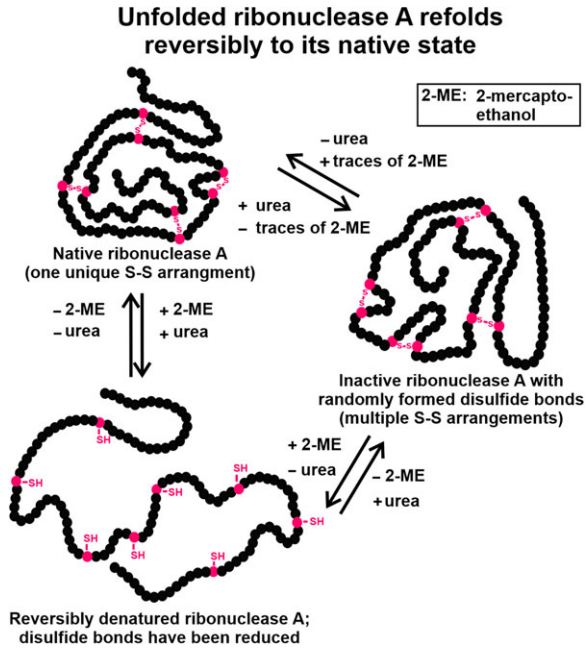

Fig. 2 Ribonuclease A folds reversibly to its native state. Anfinsen's well-known experiments on ribonuclease A showed that the native state of a protein is determined solely by its sequence, within a given environment, including a specific temperature, pressure, pH and buffer conditions (Haber and Anfinsen, 1962; Anfinsen, 1973). Adapted from Principles of Biochemistry fourth ed., Pearson Prentice Hall Inc., 2006

by Levinthal lead only to very high-chemical-potential species, the folding of most proteins is generally believed to be under thermodynamic control.

Kinetic trapping relative to intermediate and unfolded states

Although most proteins are known to follow Anfinsen's thermodynamic hypothesis, there are a few that fold under kinetic control (Baker and Agard, 1994; Eder and Fersht, 1995). An interesting example is α-lytic protease (Fig. 3A), which has a folding intermediate that is kinetically trapped as a result of high free-energy barriers unless a covalently bound pro region, which serves as a folding catalyst, is present (Baker and Agard, 1994). A representative chemical-potential landscape for this protein is provided in Fig. 3B. The pro region also serves the purpose of stabilizing the folded state, thus thermodynamically favoring its formation. The pro region is then cleaved, to give rise to a kinetically trapped, yet biologically active, native state (Kelch *et al.*, 2012).

Another example is that of proteins from the serpin family (Fig. 3C), which initially fold into an active state but slowly convert into a stable, inactive conformation over time (Fig. 3D) (Hekman and Loskutoff, 1985; Baker and Agard, 1994).

In summary, proteins have, in most cases, evolved to allow the unfolded state to convert into the bioactive native state under thermodynamic control.

However, in contrast with kinetic trapping relative to unfolded or intermediates, a protein may also be trapped relative to its aggregated states.

This review highlights the latter concept in the case of both individual purified proteins and most *E. coli* soluble proteins. As discussed below, it is becoming apparent that a broader view of Anfinsen's thermodynamic hypothesis is necessary to properly take into account the multiple states of proteins (native, unfolded, intermediates and aggregates).

Kinetic trapping relative to aggregates

Large-scale aggregation of proteins—whose biological function requires monomers or monodisperse lower-order supramolecular assemblies—is often undesirable (Amani and Naeem, 2013). On one hand, aggregates decrease the native-state population, thus perturbing net activity and proteostasis. In addition, aggregates may be toxic to the cell (Amani and Naeem, 2013). The case of protective or functional aggregates is beyond the scope of this review (Deshmukh *et al.*, 2018).

The role of aggregation in protein folding began to be acknowledged in early studies by the Oliveberg group, which showed that self-associated protein states are sometimes formed transiently during folding and then progressively convert to the native state (Silow and Oliveberg, 1997). Later on, a number of investigations reported the concurrent formation of native and aggregated protein states upon *in vitro* refolding into physiologically relevant buffers (Fink, 1998; Chiti, *et al.*, 2002; Fandrich *et al.*, 2003; Dobson, 2004; Chow, *et al.*, 2006; Jahn and Radford, 2008; Neudecker, *et al.*, 2012).

In 2001, pioneering experiments in the Prusiner lab showed that the recombinant, monomeric and α-helical prion protein is kinetically trapped relative to the more thermodynamically stable β-sheet aggregated isoform (Baskakov *et al.*, 2001). In 2002, Gazit suggested that the folded state of proteins may be metastable relative to aggregates and that, after a protein has reached its folded state, the kinetic flux (i.e. the rate) for the interconversion between fully-folded bioactive proteins and aggregates may be small (Gazit, 2002). A few years later, Baldwin and coworkers showed that some small, amyloid-prone proteins (<150 amino acids) exceed their critical concentration for aggregation *in vivo*, and that amyloid fibril formation should occur if the folding/aggregation process were under thermodynamic control. However, no aggregation is, in practice, observed under physiologically relevant conditions (Baldwin *et al.*, 2011; Thirumalai and Reddy, 2011). Therefore, the authors

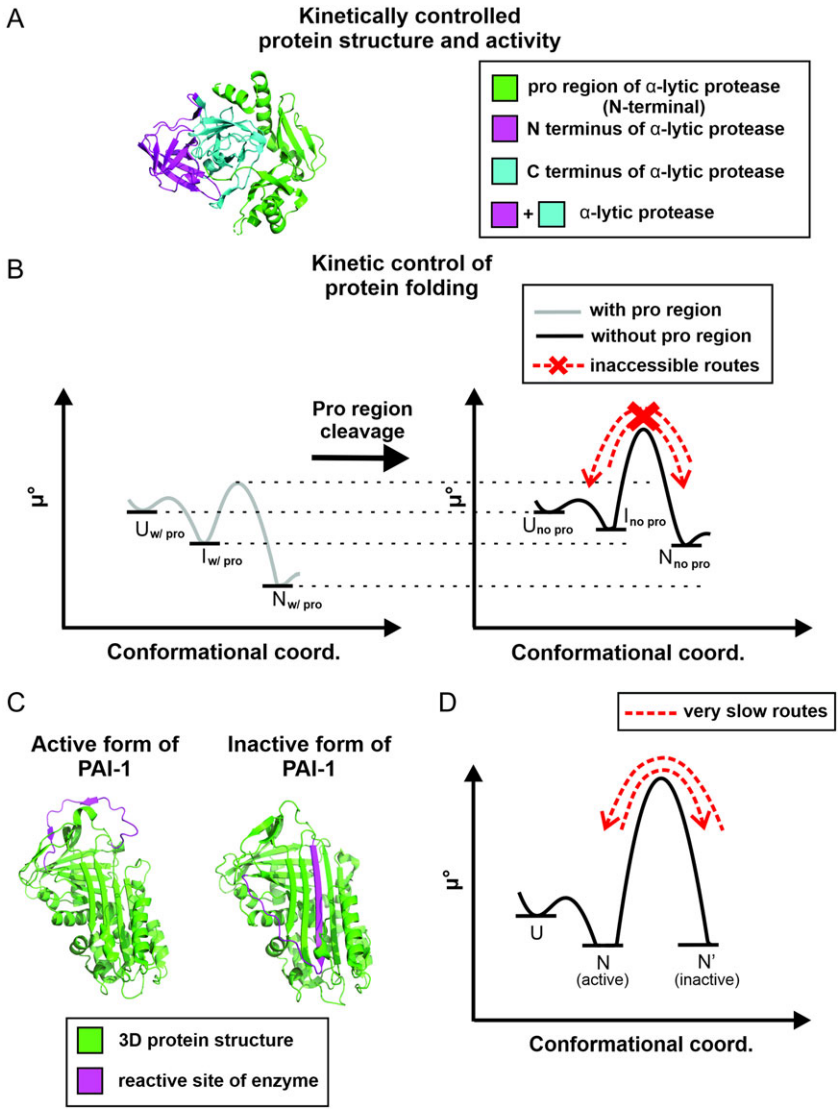

Fig. 3 Some proteins fold under kinetic control. (A) Crystal structure of α-lytic protease (magenta and cyan, PDB code: 4PRO) in complex with a noncovalently bound pro region (green). **(B)** The unfolded (U) state of α-lytic protease turns into an intermediate (I) that is unable to reach its native state (N) unless it is covalently linked to an N-terminal pro-region. The pro region serves as a *built-in* catalyst: it significantly decreases the activation barrier for folding and stabilizes the native state (N). **(C)** Crystal structure of the active (PDB: 1C5G) and latent (PDB: 1DB2) forms of plasminogen activator inhibitor (PAI-1), a protein belonging to the serpin family. (**D**) PAI-1 initially folds into its native state (N) and contains a reactive loop (magenta). Over time, PAI-1 slowly converts into a stable, inactive form (N′) as the loop rearranges to generate a β-sheet structure

concluded that these small proteins are kinetically trapped relative to amyloids.

The latter concept has been more recently expanded by Varela *et al.*, who showed that the model eukaryotic protein apomyoglobin and the majority of the *E. coli* proteome are kinetically trapped relative to predominantly non-amyloid aggregates, under physiologically relevant conditions (Fig. 4) (Varela *et al.*, 2018). Specifically, apomyoglobin was characterized via a cyclic pathway that systematically changed its state from unfolded to native to aggregated, under mild solution conditions, without introducing any covalent modifications (Varela, *et al.*, 2018). Long-term incubation periods were also included to gain explicit evidence about kinetic stability. The generality of the concept of kinetic-trapping relative to aggregates was then probed upon analysis of the entire water-soluble proteome of *E. coli* bacterium.

The experiments by Varela *et al.* are unique in that they showed that native protein states can be kinetically trapped relative to aggregates that predominantly lack amyloid nature. In addition, these studies proved that the relevant aggregates can be either soluble or insoluble, depending on the total protein concentration. These findings are general in the sense that (a) they apply to both eukaryotic apomyoglobin and to the majority of the proteins in the *E. coli* proteome, and (b) are applicable not only to the short proteins identified by Baldwin *et al.* (Baldwin, *et al.*, 2011) but also to polypeptides and proteins of a wide range of molecular size. Most of the proteins (70–80%) identified by Varela *et al.* were found to be kinetically trapped on timescales longer than the *E. coli* lifetime, at room temperature and at concentrations much lower than intracellular values.

Computer simulations by Varela, *et al.* (2018) showed that the protein kinetic trapping relative to aggregates is not necessarily due

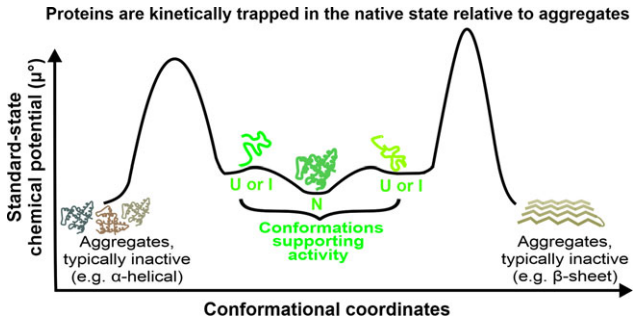

Fig. 4 The majority of *E. coli* proteins are kinetically trapped relative to aggregates. Schematic representation of the main conclusions of the study by Varela *et al.* (2018). Protein unfolded and native states, as well as folding intermediates, are denoted as U, N and I, respectively. In general, a wide variety of aggregated states are possible, encompassing different secondary structure and a wide range of particle sizes

to the presence of high kinetic barriers. Both protein concentration and kinetic-barrier height for the aggregation rate-determining step contribute to the observed aggregation flux (i.e. rate) for the formation of aggregates starting from the native, intermediate or unfolded state. Therefore, even proteins characterized by small kinetic barriers for aggregation can be kinetically trapped, as long as their concentration is very low.

Kinetic trapping of the native state relative to self-associated states can be eliminated when the native state is incubated in solution in the presence of aggregates of specific secondary structure. For instance, β-sheet but not α-helical aggregates of apomyoglobin are able to promote the slow aggregation of the monomeric native state of this α-helical protein (Varela, *et al.*, 2018). In addition, smaller incompletely elongated aggregates are computationally predicted to be more effective seeds than larger aggregates (Varela, *et al.*, 2018). On the other hand, the pure folded protein persists as a kinetically trapped native state, upon long-term incubation.

In all, Varela *et al.* found that there are three basic ways to 'kinetically untrap' a protein from its native state, namely (i) incubation in the presence of selected aggregates of specific secondary structure, (ii) increases in protein concentration and (iii) higher temperature. Other environmental conditions such as pH, buffer composition and the presence of molecular crowders and(or) osmolytes may also well play a role, though additional studies are necessary to explore this topic.

In summary, many folded proteins are kinetically trapped relative to aggregated states under physiologically relevant conditions.

Protein kinetic partitioning and trapping upon release from the ribosome

Recent studies (Addabbo *et al.*, submitted) found that, immediately after translation termination, newly synthesized proteins kinetically partition between the fully folded native state and aggregated conformations. The basic aspects of this process are schematically illustrated in Fig. 5. This partitioning is typically successful for wild-type proteins, which consistently attain their native state with much higher yield than upon *in vitro* refolding from chemically denatured states. The presence of molecular chaperones enhances the yields of native state upon release from the ribosome. The above kinetic partitioning is extremely sensitive to amino acid sequence. As a consequence, single-point mutations (that have no effect on native-protein stability and tertiary structure) can easily tilt the balance in favor of aggregation.

The above investigations also showed that, after a wild-type model protein has been released from the ribosome and quantitatively converted to the native state, it remains kinetically trapped in that state (relative to aggregates) over long timescales (Fig. 5).

In the case of *de novo*-synthesized mutant proteins that are produced as a mixture of native state and aggregates, the scenario is more complex. The native state and soluble-aggregates were found to persist with time, while the insoluble aggregates progressively turned into a soluble population, possibly due to the action of disaggregases (Diamant *et al.*, 2000; Zblewska *et al.*, 2014; Mogk *et al.*, 2018).

In short, when newly synthesized proteins are produced with no aggregates, they preserve their native conformation and remain aggregate-free over long timescales. However, when the newly synthesized population includes aggregated states, further aggregation may occur over time.

Kinetic trapping in nature

Kinetic trapping under physiological conditions is not a unique characteristic of protein folding and aggregation. For instance, unimolecular kinetic trapping in RNA folding is a well-known phenomenon (Treiber and Williamson, 1999). In addition, in aqueous solution, polysaccharides (Wolfenden *et al.*, 1998), polypeptides (Radzicka and Wolfenden, 1996; Martin, 1998), proteins (Radzicka and Wolfenden, 1996), DNA (Radzicka and Wolfenden, 1995) and RNA (Thompson and Fisher, 1978) are usually kinetically trapped—as well as thermodynamically less stable—relative to the corresponding hydrolytic reaction products. Therefore, the concept of kinetic trapping is widespread in Nature, and it applies to processes involving both covalent and noncovalent chemistry. Material science is also characterized by numerous instances of kinetic trapping at ambient temperature and pressure, including glasses (Angell, 1991), perovskites (Chen *et al.*, 2016), nanoparticles (Grammatikopoulos *et al.*, 2016) and self-assembled nanomaterials (Yan *et al.*, 2016).

The familiar process of egg cooking

Several common every-day-life events are governed by kinetic trapping at the molecular level, e.g. the well-known process of egg cooking at high temperature followed by cooling. Given the recurrent nature of this phenomenon, we will summarize its major features. For simplicity, we focus only on comparisons with the egg albumen (Shimada and Matsushita, 1980; Shigeru and Shuryo, 1985; Mine *et al.*, 1990; Van der Plancken *et al.*, 2005, 2006; Croguennec *et al.*, 2007), as the yolk is more complex.

The most abundant protein in egg white is ovalbumin. This protein contains one disulfide bridge and four sulfhydryl groups in its native state at room temperature (Thompson and Fisher, 1978; Nisbet *et al.*, 1981). Within the egg albumen, ovalbumin is known to undergo extensive disulfide bridge rearrangements upon heating followed by cooling in an oxygen-containing atmosphere (Van der Plancken, *et al.*, 2005, 2006). Heating was found to facilitate the formation of large soluble and insoluble aggregates across the 7.2–7.6 pH range (Mine, *et al.*, 1990; Van der Plancken, *et al.*, 2005; Yang *et al.*, 2015). Aggregate formation upon heating is known to occur for many other proteins (Van der Plancken, *et al.*, 2006), consistent with earlier sections of this review.

However, the formation of new disulfide bridges occurs concurrently or shortly after nonpolar surfaces have been exposed upon unfolding (Van der Plancken, *et al.*, 2006). Aggregation is therefore believed to be driven by solvent exposure of nonpolar groups at high temperature, rather than by disulfide exchange (Mine, *et al.*,

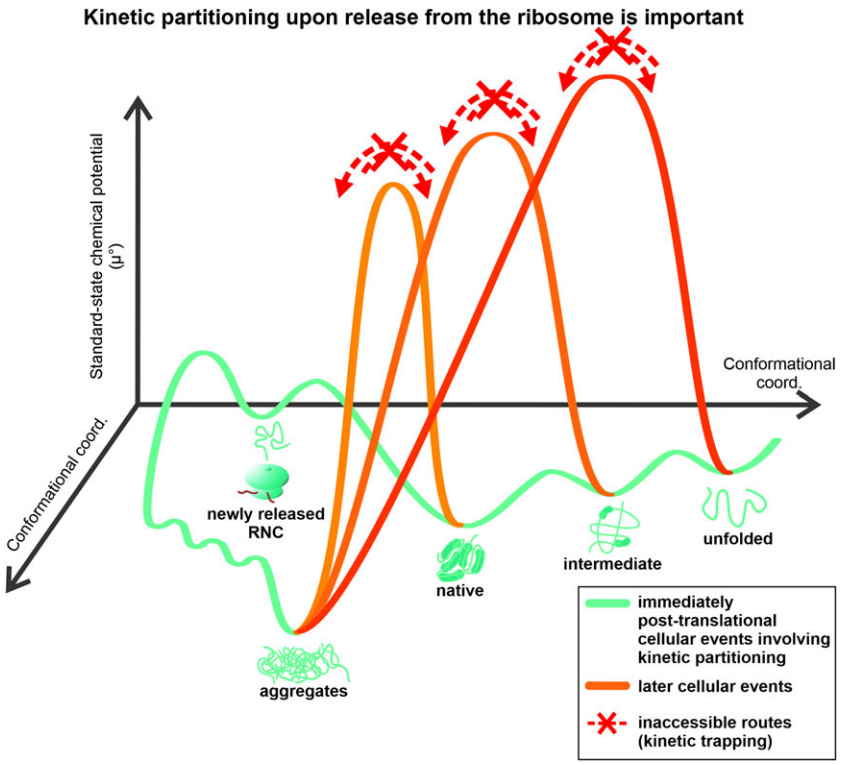

Fig. 5 Chemical-potential landscape of cellular proteins upon release from the ribosome under physiologically relevant conditions. Protein folding upon release from the ribosome typically involves kinetic partitioning between routes leading to native and aggregated states. This process is usually successful for evolutionarily optimized proteins, leading to the quantitative formation of the native state. In general, the presence of molecular chaperones increases the population of native state upon release from the ribosome. After the above immediately-post-translational kinetic partitioning has taken place, the native, intermediate and unfolded states of apomyoglobin remain kinetically trapped relative to aggregates. The standard-state chemical-potential landscape (on a per-monomer basis) shown in this diagram, and already adopted elsewhere (Varela *et al.*, 2018), is a convenient representation because the features of this diagram are concentration-independent and enable direct comparisons between monomers and aggregates

1990). On the other hand, disulfide exchange, which occurs shortly after hydrophobicity-induced aggregation (Van der Plancken, *et al.*, 2006), reinforces the gel-like properties of heated-and-cooled egg albumen, and likely enhances the thermodynamic stability of the aggregates (Mine, *et al.*, 1990). Similar effects have been reported for other proteins (Yang, *et al.*, 2015).

Interestingly, the heated-and-cooled ovalbumin aggregates display an enhanced β-sheet secondary-structure content (Mine, *et al.*, 1990), similarly to the heating and cooling of cysteine-free apomyoglobin (and many *E.* coli proteins under reducing conditions) discussed in previous sections (Varela *et al.* 2018). In all, the available experimental data underscore the fact that formation of insoluble β-sheet-enriched aggregates upon heating-and-cooling is not merely a consequence of disulfide-bridge formation.

In summary, the kinetic trapping of protein native states (relative to aggregates) and the familiar process of egg cooking clearly have a lot in common. Yet, experiments on model proteins lacking disulfide bridges, e.g. apomyoglobin, described in the previous sections (Varela, *et al.*, 2018), enable drawing more clear-cut conclusions on the nature of non-covalent aggregation, given that disulfide-bond formation and/or reshuffling can be explicitly factored out.

Concluding remarks

Overall, the research discussed in this review highlights the need to expand Anfinsen's thermodynamic hypothesis to include non-amyloid (Varela, *et al.*, 2018) as well as amyloid (Baldwin, *et al.*, 2011) protein aggregates. The native state may either be trapped relative to intermediates, the unfolded ensemble or soluble/insoluble aggregates (Baker and Agard, 1994; Baldwin, *et al.*, 2011; Varela, *et al.*, 2018). When kinetically inaccessible conformations are poorly populated and more thermodynamically stable than the native state, the kinetically-trapped native state is metastable. Kinetic trapping of the native state relative to soluble and insoluble aggregates was found to apply to a large variety of proteins, including most of the *E. coli* bacterial proteome (Varela, *et al.*, 2018). Interestingly, the aggregates can arise from both small and large proteins and do not need to have amyloid character (Varela, *et al.*, 2018). Kinetic trapping relative to aggregates is an important emerging theme in protein folding and contemporary biology.

Acknowledgments

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