Opinion TRENDS in Biochemical Sciences Vol.29 No.10 October 2004

Protein folding in the cell: reshaping the folding funnel

Patricia L. Clark

Department of Chemistry and Biochemistry, University of Notre Dame, 251 Nieuwland Science Hall, Notre Dame, IN 46556-5670, USA

Models of protein folding have historically focused on a subset of 'well-behaved' proteins that can be successfully refolded from denaturants *in vitro*. Energy landscapes, including folding funnel 'cartoons', describe the largely uncomplicated folding of these isolated chains at infinite dilution. However, the frequent failure of many polypeptides to fold to their native state requires more comprehensive models of folding to accommodate the crucial role of interactions between partially folded intermediates. By incorporating additional deep minima, which reflect off-pathway interchain interactions, the folding funnel concept can be extended to describe the behavior of a more diverse set of proteins under more physiologically relevant conditions. In particular, the effects of ribosomes (translation), molecular chaperones and other aspects of the cellular environment on early chain conformations can be included to account for the folding behavior of polypeptide chains in cells.

Historically, detailed investigations of protein folding processes focused on a set of small (100–200 amino acids), globular, α-helix-rich proteins that could be refolded to the native state *in vitro* after dilution from a denaturant. By contrast, proteins that formed inactive insoluble aggregated states after dilution, such as tubulin, actin, T4 DNA polymerase, phosphoglycerate kinase [1], collagen [2] and many polypeptides with all-β-sheet topologies, were excluded from the initial models of protein folding. The limitations of these early simple models became clear when the biotechnology industry began to encounter problems during the production of therapeutically important proteins. Many of these proteins failed to fold into their native states in cells and instead accumulated in an inactive aggregated inclusion body state [3,4].

As the expression of proteins in bacteria and other hosts has expanded to incorporate a wider range of proteins, the failure of protein folding and the accumulation of insoluble states have emerged as a common phenomenon. Many cell biologists, having been taught that polypeptide chains can spontaneously fold to the native state, have been frustrated to discover that, although spontaneous folding can occur for small simple proteins such as RNase A, spontaneous, high-yield folding to the native state might be the exception, rather than the rule, especially when proteins are overexpressed at high levels. In fact, spontaneous folding is not an absolute certainty even for RNase A, as Anfinsen and coworkers [5] documented in 1961. If RNase A concentrations are increased to mimic physiological concentrations, there is a marked drop in the yield of native protein.

In subsequent studies, Anfinsen and coworkers showed that partially folded chains become trapped in an aggregated state with incorrect disulfide bond pairings. Their observations led to the search for and identification of the first protein disulfide isomerase [6]. Nevertheless, despite the ubiquity of competing off-pathway aggregation reactions, aggregation in protein expression and folding is still largely viewed as a nuisance, rather than as a physiologically relevant pathway in its own right.

The discovery that molecular chaperones have an essential role in the recognition and/or dissociation of protein folding intermediates in danger of self-association has firmly established that the competition between productive folding and aggregation is a fundamental feature of folding in cells [7,8]. Furthermore, since the discovery that numerous human diseases are caused by protein aggregation, the physiological roles of misfolded and polymerized states have been receiving increased attention [9]. With this increased recognition of the role of aggregation in human disease and the desire to express a wide range of proteins in bacteria and other hosts, it has become important that theoretical models encompass the full range of protein folding, aggregation and amyloid fibril deposition processes. Unfortunately, there is still a gap between popular theoretical formulations of protein folding processes and experimental data on the behavior of physiologically important proteins in cells. Here, an attempt is made to bridge this gap in a manner that might be useful to computational biologists, protein biochemists, structural genomicists and cell biologists.

Characteristics of protein aggregates

Investigations into the fates of newly synthesized polypeptides *in vivo* have revealed that inclusion body aggregates are typically formed from partially folded conformations, rather than from native states or fully denatured polypeptide chains [3,4]. Formation of an aggregated state *in vitro* also represents the polymerization of partially folded intermediates [10,11]. These reactions are specific; in a mixture of refolding proteins,

Corresponding author: Patricia L. Clark (pclark1@nd.edu).

 0968-0004/$ - see front matter doi:10.1016/j.tibs.2004.08.008

partially folded chains typically aggregate with their own species and do not cross polymerize or aggregate.

For a few polypeptides, we have some understanding of the molecular basis of the competition between productive folding and aggregation. For example, native collagen triple helices are aligned in exact register. On denaturation and refolding, however, the collagen polypeptide chains can form gelatin, rather than native collagen. Specifically, the chains form local regions of collagen-like triple helix, but the mature chains alone also form a stable 3D network of intermolecular interactions. Careful control of experimental conditions is required to unfold and refold mature collagen reversibly, and the process is extremely slow. The information in the collagen sequence is therefore insufficient to refold collagen to its native structure on a physiologically relevant time scale. In the cell, each collagen polypeptide chain is synthesized with a specific C-terminal sequence that directs the registration process, but these sequences are cleaved during export of collagen to the extracellular space and are absent in the mature protein [12].

Another well-characterized example is provided by the aggregates formed by the loop–sheet insertion mechanism of serine protease inhibitors (serpins) such as α_1-antitrypsin. For wild-type α_1-antitrypsin, cleavage of the inhibitory loop by a target protease results in insertion of this intramolecular loop sequence into the central β-sheet of α_1-antitrypsin. In individuals carrying the α_1-antitrypsin Z allele (Glu342→Lys), however, the loop can insert prematurely (before cleavage) and intermolecularly, forming polymers that disrupt the endoplasmic reticulum during maturation in the liver. These aggregates are very stable and do not dissociate under physiological conditions [13]; moreover, similar to the native protein, the aggregated chains have specific conformational features including the loop insertion motif. These polymers might persist only because of the depth of their kinetic trap (i.e. the large activation energy required to convert a subunit back into its native monomeric structure, see below); alternatively, the polymer conformation might be more stable than the native state.

A high-resolution structure of amyloid fibrils is not available as yet, but the peptide components are known to adopt a β-strand conformation that, although specific, is distinct from the conformation of the soluble native precursor proteins [14,15]. Amyloid fibers are very stable and do not spontaneously dissociate to monomers; in some cases, the fibers are more stable than the soluble native state, which itself might have only marginal stability [16].

Features and limitations of current folding funnels

A popular theoretical formulation adapted by the protein folding community is the energy landscape perspective, often represented as a 'folding funnel' cartoon, that describes the *in vitro* progression of an isolated polypeptide chain from an ensemble of denatured, random conformations to the native structure at the global energy minimum (Box 1). Folding funnels were developed to describe the refolding of peptides and short polypeptide chains from denaturants and do not attempt to account for

Box 1. Folding funnels are commonly used to describe the folding of 'well-behaved' proteins *in vitro*

A folding funnel is a simplified 2D or 3D representation of the very high-dimensional conformational space that is accessible to the polypeptide backbone during folding [25]. Energetically, the breadth of the funnel represents all possible conformations of the chain (chain entropy): the broad top of the funnel depicts the enormous number of conformations present in the soluble denatured state, such as an ensemble of starting conformations populated on rapid dilution from a urea- or guanidine-denatured state; the needle-like point at the bottom of the funnel represents the unique native structure of the protein as determined by X-ray crystallography or NMR. The separation between the top and bottom of the funnel represents other energy contributions (chain enthalpy, solvent entropy and enthalpy) to each chain conformation.

In the coordinate system shown in Figure I, each point on the funnel surface represents a specific possible conformation of the polypeptide chain and its corresponding energy value. The sloping sides of the funnel represent folding pathways for individual starting conformations (indicated by arrows in Figure I) of the polypeptide chain. This is a key difference between the representation of protein folding as a funnel and its representation as a classic chemical reaction coordinate. In a chemical reaction coordinate, all reactants proceed through one pathway with an identical transition state or states and intermediates, if present. By contrast, the folding funnel allows several routes to the native structure – an appropriate model, given the enormous conformational heterogeneity in the starting material.

As the chain folds to lower energy conformations, it might populate intermediate states, indicated by local minima (I_A and I_B) along the sides of the funnel. These kinetic traps might hinder and/or promote formation of the native structure depending on their depth, the barriers between the trap and the native conformation, and the rest of the funnel surface. In the terminology of statistical mechanics, the number and depth of local kinetic traps on the funnel landscape represent the degree of frustration of the polypeptide sequence [55]. The ability of a specific trap to hinder or to promote native state folding can be evaluated by considering how water would run down the surface of the funnel [25]. The fraction of polypeptide chains that do not fold correctly and aggregate is presumed to be small and is therefore ignored.

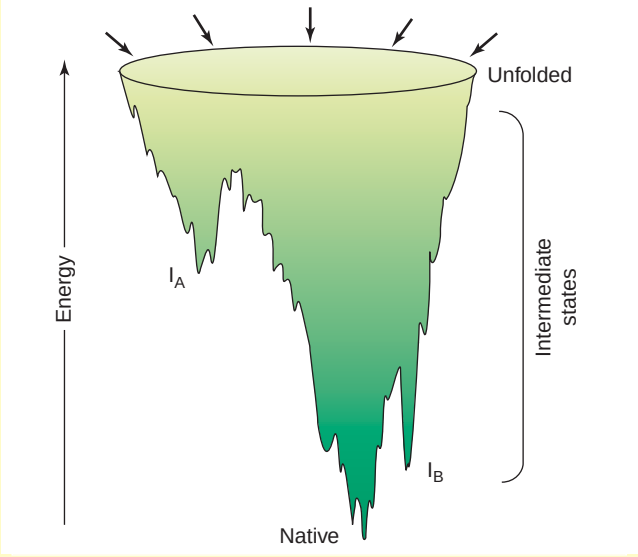

Figure I. A typical folding funnel diagram used to describe the folding of a well-behaved (often single-domain) protein *in vitro*.

the behavior of newly synthesized polypeptide chains released from ribosomes in cells. Folding funnels are an appealing representation for modeling the folding behavior of string models, small peptides, simple heteropolymers and glass transitions under a limited set of experimental conditions [17,18].

Current folding funnels cannot, however, describe the behavior of most polypeptide chains under physiological conditions. Although the models start with all possible initial conformations at the top of the funnel, they describe the folding behavior of only a single polypeptide chain at infinite dilution. They do not consider populations or incorporate realistic intermolecular collision frequencies. As a result, an intrinsic feature of actual folding processes – namely, collisions between partially folded chains that lead to self-association – is excluded from consideration. Because misfolding is often associated with self-association, polymerization or aggregation, the current funnel models cannot account for the aggregation behavior of many proteins that have been well studied in the biotechnology industry, including insulin, bovine growth hormone, tissue plasminogen activator and granulocyte colony stimulating factor [19,20].

A few research groups have begun modeling aggregation processes by modeling chain folding as two (or more) strings that are allowed to interact. Although much more work is needed in this area, an immediate result has been the identification of dominant pathways for folding and aggregation [21–23]. In this article, the folding funnel formulation has been extended to include funnels with additional deep minima, which turns out to be useful in appreciating not just the off-pathway aggregation reaction, but also the roles of chaperones, ribosomes and other cellular factors that function, presumably, to prevent chains from populating conformations that self-associate into kinetically trapped states *in vivo*. This extends funnel cartoons to a form that represents more accurately the actual range of protein folding and aggregation phenomena that has been documented for both prokaryotic and eukaryotic proteins.

Adding alternative deep minima to describe competing aggregation reactions

Consider a highly simplified aggregation reaction (Figure 1a): a partially folded polypeptide chain forms about 50% of the intramolecular contacts present in the native structure. Instead of forming additional native contacts, however, the chain might collide with another polypeptide chain in an identical conformation, forming additional, non-native stabilizing contacts between the two polypeptide chains. This simplified 'dimer aggregate' could be similar to the conformation, for example, of a domain-swapped dimer [24].

In folding funnel diagrams, an off-pathway aggregation reaction can be incorporated either as a second contour on a higher-dimensional energy landscape [Figure 1b(i)] or, more simply, as a second 'aggregation' funnel that is relevant to aggregation-prone conformations of the polypeptide chain and represents the interactions of partially folded intermediates into a self-associated or polymerized native state [Figure 1b(ii)]. This example is highly simplified: in reality, most protein aggregates are larger, higher-order multimers, and intermolecular stabilization on the aggregation pathway can presumably occur between chains in non-identical conformations. These considerations complicate the drawing of double funnel diagrams, but even the simplified example shown in Figure 1b is useful for considering the effects of aggregation on productive folding processes and cellular mechanisms for avoiding aggregation.

This 'double funnel' concept is supported by experimental evidence that indicates that protein aggregates – even so-called 'amorphous' aggregates – form from the assembly of specific partially folded intermediates and are further stabilized by interactions between these intermediates [3,4]. The deep minimum in the aggregation

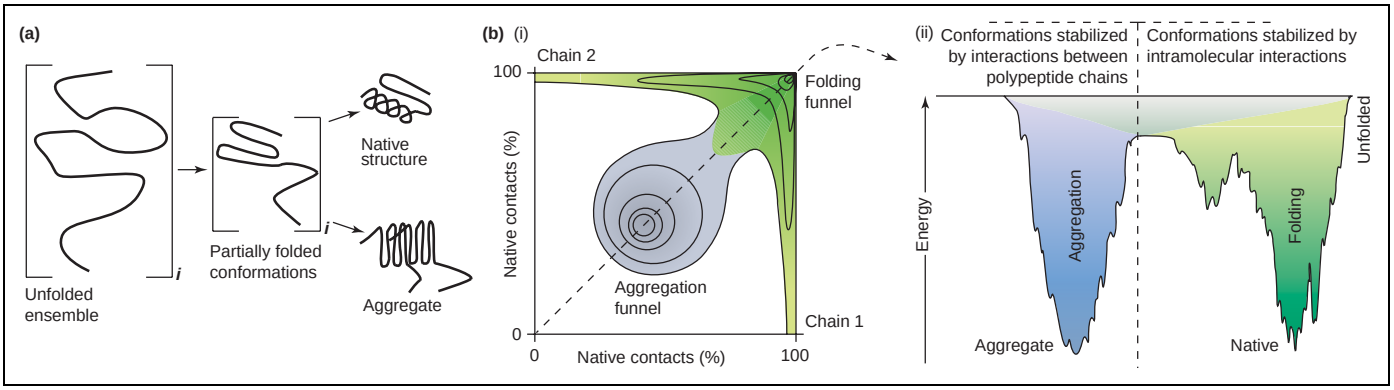

Figure 1. For most proteins, folding to the native state occurs concomitantly with misfolding and aggregation. **(a)** The simplified *in vitro* refolding and aggregation processes for a pool of identical polypeptide chains. From an initial large ensemble of random conformations, partially folded conformations are formed. The latter species might continue to fold to the native structure; conversely, they might misfold, associate with other copies of the polypeptide chain, and aggregate. **(b)** Folding funnels designed to describe the competition between productive folding and aggregation. (i) A higher-dimensional energy landscape contour plot that includes stabilizing interactions between two polypeptide chains. In this simplified example, the stable aggregate is formed via intermolecular stabilizing interactions that develop between two partially folded conformations with about 50% of the contacts present in the native structure. Darker colors represent lower energy conformations. (i) A double funnel depicting the competition between folding and aggregation; in a simplified sense, this double funnel cartoon is related to a projection of the diagonal (broken line) on the contour plot. Conceptually, the aggregation funnel includes the accumulation of interactions between several chains present in the aggregated state. Note that the aggregation funnel could also contain populated intermediate states, as seen in the folding funnel. The lowest energy state for the aggregate might or might not represent the true global energy minimum; regardless of this, it is sufficiently kinetically trapped from the native conformation that as to make this distinction difficult to determine. The double funnel highlights the important roles of both early chain conformations (the chain distribution around the funnel top) and the height of the barrier separating the two funnels in distinguishing between productive folding and aggregation pathways.

funnel therefore represents the intersection of several energy landscapes for individual polypeptide chains (two chains in this simplified example) and is fundamentally different from the behavior of one chain at infinite dilution. It is important to note that it is very difficult to draw simple cartoons that capture the complexities of the physics involved in these intermolecular processes; nevertheless, the highly simplified double funnel shown in Figure 1b does succeed in capturing many fundamental concepts. For example, cartoons such as this can be used to suggest the shape (breadth and depth) of an aggregation funnel.

An aggregation energy minimum might be poorly defined; alternatively, it might be as well defined as, or even better defined than, the native state minimum. For proteins such as α-synuclein, which does not adopt a stable native structure in solution but becomes highly ordered on polymerization [16], the energy minimum for the aggregation funnel might be deeper and/or sharper than that for the folding funnel. Conversely, if a protein can adopt various misfolded polymerized states, the aggregation funnel might be very broad, although so far studies suggest that many aggregating chains adopt a well-defined, ordered conformation even in 'amorphous' aggregates [3,4]. In addition, plots such as those in Figure 1b can highlight the structural relationship between the conformations of an aggregation precursor and those of the native structure. It is tempting to extend this formulation further to describe the concentration dependence of aggregation, but such a discussion is beyond the scope of this article.

The inclusion of a competing aggregation pathway represents more accurately the full range of fates for folding polypeptide chains and clarifies the true complexity of protein folding…

The presence of multiple funnels in a single energy diagram has been proposed previously (e.g. see Ref. [25]) and has been demonstrated in the calculated energy landscape of a model peptide with two stable low-energy states [26]. With few exceptions (e.g. see Ref. [27]), however, the effect of intermolecular associations on the energy landscape has not been explored, despite the key role that intermolecular interactions have in biological processes. The inclusion of a competing aggregation pathway represents more accurately the full range of fates for folding polypeptide chains and clarifies the true complexity of protein folding, which includes describing not only how a protein folds, but also why a protein does not aggregate.

The double funnel diagram also draws attention to experimental results describing how 'aggregation avoidance' is encoded in the amino acid sequence of a protein. For example, the isolation of point mutations that function as global suppressors of aggregation provides direct evidence that the amino acid sequence both stabilizes the native pathway and inhibits off-pathway interactions [28]. Likewise, many membrane proteins contain a proline residue in the middle of a transmembrane α-helix [29]. By most criteria, proline would disfavor folding because it destabilizes the native helix structure. For the cystic fibrosis transmembrane conductance regulator, however, the proline residue disfavors alternative conformations of the transmembrane segment, such as β-strands, that lead to aggregation. Hence, the mid-helix proline residue is not just tolerated but evolutionarily conserved [29].

Similarly, a recent study has shown that the β-strands at the edges of β-sheets have unique characteristics. Simply put, the polypeptide chains of β-sheet proteins seem to have evolved to include edge strand features such as proline residues, β-bulges and capping loops that prevent the β-sheets from forming incorrect intermolecular associations [30]. In the context of the double funnel diagrams shown in Figure 1b, aggregation avoidance can be considered as strategies used to bias the polypeptide chain conformational ensemble away from aggregation-prone precursor conformations, which would constrict the top of the aggregation funnel.

The effects of Hsp60-type molecular chaperones on protein folding

A small but significant fraction of proteins, including the well-studied examples of tubulin and ribulose 1,6-bisphosphate carboxylase/oxygenase (RuBisCO), fail to fold to the native state in the absence of Hsp60, a cylindrical molecular chaperone often associated with the cellular response to heat shock. Lorimer and colleagues [31], in their original characterization of the rescue of RuBisCO refolding by GroEL/ES (the *Escherichia coli* Hsp60 chaperone system), established that this bacterial chaperonin recognizes a partially folded intermediate at risk of self-association and assists in getting the intermediate past this junctional conformation.

By including the aggregation reaction and its conformations in the energy landscape for folding, the role of Hsp60 chaperones in protein folding can be represented more clearly. Considerable work over the past two decades has shown that Hsp60 interacts with partially folded intermediates, particularly those with an exposed hydrophobic surface area, and can markedly shift the distribution between productive folding and aggregation. In the context of a double funnel diagram, Hsp60 binding to partially folded polypeptide chains can be viewed as a recognition event that occurs at a discrete zone near the junction between productive folding and aggregation (Figure 2).

Currently, the precise effects of molecular chaperones on the energy landscape of a folding polypeptide chain have been described by two working models: the so-called 'iterative annealing mechanism' and the 'Anfinsen cage mechanism' [32]. Both include a cycle of polypeptide chain binding and release driven by ATP hydrolysis, but they disagree on how much folding occurs in the central cavity of Hsp60 and how much Hsp60-induced unfolding occurs. Regardless of these differences, in the double funnel diagram, Hsp60 binding can be visualized as the

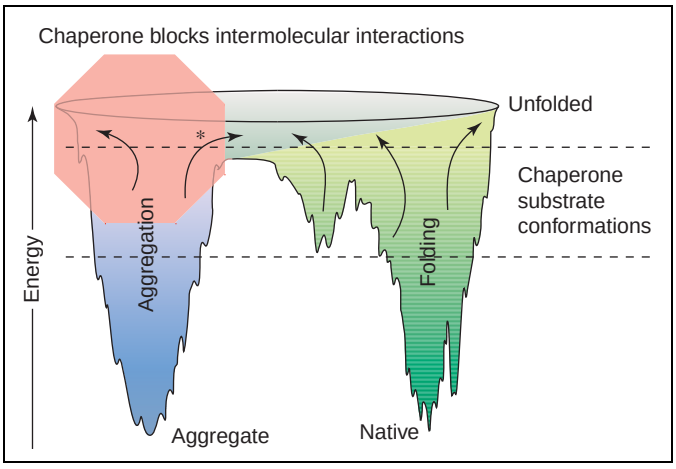

Figure 2. The effect of Hsp60 molecular chaperones on chain conformations in the context of a multiple-minima folding funnel. For simplicity, funnel space is shown only for stabilizing conformations. Hsp60 molecular chaperones recognize polypeptide chains (in either funnel) with conformations that expose significant amounts of hydrophobic surface area; these conformations are indicated by the area of the funnels within the broken lines. In the iterative annealing mechanism, the ATP hydrolysis cycle releases polypeptide chains with fewer stabilizing interactions or higher chain entropy (i.e. a higher position on the funnel surface, indicated by the curved black arrows), permitting a new path down the funnel surface that possibly traverses the barrier separating the folding and aggregation funnels (arrow marked with asterisk). In the Anfinsen cage mechanism [32], chain isolation in the central cavity of the chaperone effectively blocks the aggregation funnel (i.e. the conformations stabilized by intermolecular interactions, indicated by the pink octagon); this portion of the energy landscape becomes inaccessible to the chain.

recognition of a partially folded chain in either the folding or the aggregation funnel.

The iterative annealing mechanism suggests that Hsp60 might catalyze the rearrangement of this conformation to a less-ordered conformation at a 'higher' point on the folding funnel. Although chaperone-mediated rearrangement could slow down the bulk folding rate, rearrangement of a kinetically trapped conformation could provide the polypeptide chain with the opportunity to refold more quickly through an 'untrapped' trajectory on the folding funnel, which could have the net effect of 'smoothing' the energy landscape – a concept that is supported by recent molecular dynamics simulations [33]. Conversely, the Anfinsen cage mechanism [32] suggests Hsp60 binding might isolate the chain from intermolecular interactions that lead to aggregation, thereby passively promoting folding by blocking access to the aggregation funnel. Folding in the Hsp60 cavity could provide many of the positive benefits (promotion of compaction and folding) of macromolecular crowding, while at the same time avoiding the negative effects (increased aggregation) of crowding in the highly concentrated cell cytoplasm [34].

Intervention by molecular chaperones could be particularly important for proteins with native structures that do not represent the thermodynamic global energy minimum. Chaperones could have an integral role in kinetically trapping these chains into compact functional structures, similar to the way in which the prodomain of α-lytic protease, an intramolecular chaperone, traps the mature protease in a conformation that is not the global energy minimum [35]. Indeed, the assumption that the active, native structure is the global energy minimum conformation might be an oversimplification. Perhaps many native states are maintained not because they are the thermodynamically lowest energy state, but rather because cellular circumstances have left them kinetically trapped in that state [35]. What, then, represents the true minimum energy structure for these proteins? For some polypeptides, the aggregated state might be the lowest energy state.

Folding funnels for elongating nascent polypeptide chains

The full spectrum of cellular proteins includes very long proteins, intertwined multimeric complexes, and proteins with substantial posttranslational modifications. Although many of these polypeptides are highly prone to aggregation during refolding in the test tube, <20% of cytoplasmic proteins seem to require interactions with molecular chaperones to achieve correct folding *in vivo* [36]. This means that >80% of newly synthesized cytoplasmic polypeptide chains seem to be capable of folding unassisted [37,38]; nevertheless, of those chains that have been examined in detail, folding is typically still more efficient *in vivo* than *in vitro* [39,40].

In addition to molecular chaperones, protein folding in the cell incorporates other features that are not present during refolding *in vitro*. For example, newly synthesized polypeptide chains do not appear all at once, but rather make a vectorial appearance in the cytoplasm, cell membrane or other compartment. In addition, the chains do not fold in a dilute homogeneous solution at low temperature, but at a high concentration in an extremely heterogeneous environment, which is often at a much higher temperature than those used for *in vitro* refolding; in other words, the presence of the competing aggregation funnel looms large on the energy landscape for these polypeptide chains [34].

How can we conceptualize a folding funnel for elongating chains? Consider a simple single-domain protein that can fold to its native or near-native conformation cotranslationally without the assistance of molecular chaperones or other cellular components (e.g. α-globin [41] or the N-terminal protease domain of the Semliki Forest virus capsid preprotein [42]). Early proteolysis experiments by Malkin and Rich [44] demonstrated that very short nascent polypeptide chains (<20–40 amino acids) will be contained completely within the mass of the ribosome and, presumably, will be held in a rather extended [43] or perhaps α-helical [44] conformation but with limited conformational flexibility. It seems extremely unlikely that the sequence contained in the ribosome tunnel can explore the same range of polypeptide chain conformations as can a chain in dilute solution. Thus, the starting conformations for short (<20–40 residues) nascent chains probably represent a very restricted region of conformational space and a tightly constricted portion of the funnel diagram (Figure 3).

As new amino acid residues are added to the C terminus, the chain elongates and the N-terminal segment emerges at the exit site of the polypeptide tunnel at the surface of the ribosome [45]. There is a marked increase in the accessible conformational space for the

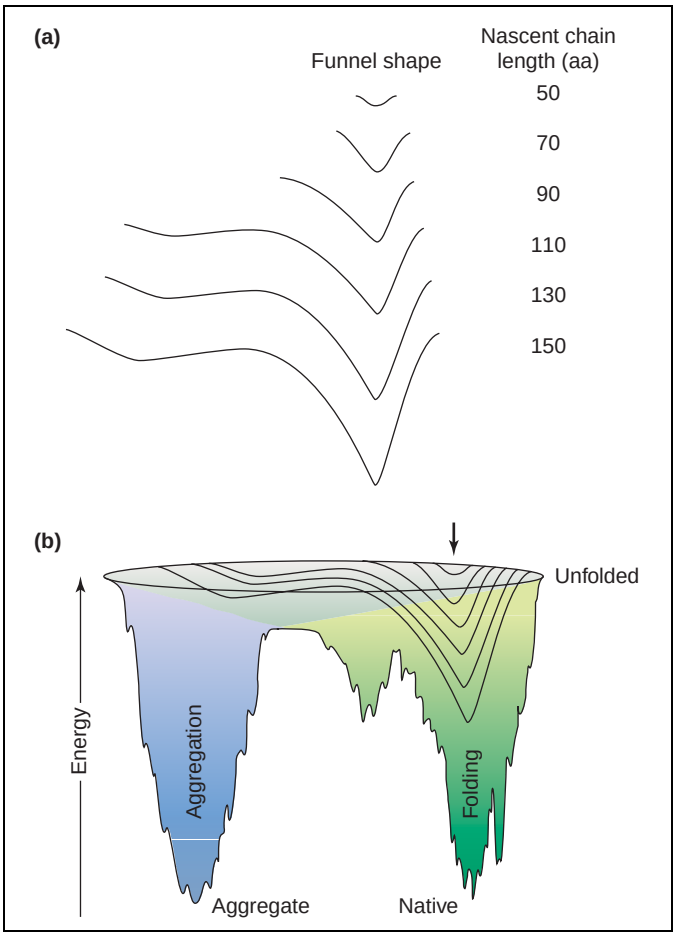

Figure 3. Folding funnels describing the folding of a newly synthesized polypeptide chain *in vivo*. In this simple example, the chain can assemble native contacts in a cotranslational manner. **(a)** Funnel shape as a function of chain length. Short chains will have both limited conformational entropy and limited energetic differences between any two conformations, and therefore their funnels will be both narrow and shallow. As the chain length grows, funnel width and depth increase, and eventually conformations prone to aggregation will be possible. After translation termination, but before chain release, the nascent chain might have access to a conformational ensemble that is close in size to (but still smaller than) the ensemble populated by *in vitro* denatured chains. **(b)** Cotranslational funnels superimposed on the *in vitro* folding and aggregation funnels for a free full-length chain. This funnel diagram indicates that the starting ensemble for cotranslational folding is not the full breadth of the funnel top, but a select subset of conformations (arrow). Early conformational bias in these shorter nascent chains is therefore expected to have a marked effect on intracellular folding yield, and might reduce the need for molecular chaperone involvement. Abbreviation: aa, amino acid.

polypeptide chain that has emerged from the exit site. Nevertheless, the flexible portion of the chain is still short and has limited conformational freedom. The 'folding funnel' for this short emerged chain is still shallow at this point: there is very little energetic separation (if any) between one conformation and another (Figure 3).

As more residues emerge from the ribosome, however, additional conformational space becomes accessible and, eventually, enough conformations will be permitted that one (or more) will be at a significantly lower energy state than the others. The cotranslational appearance of the polypeptide chain outside the ribosome therefore corresponds to a specific portion of the folding funnel, and the chain presumably folds reasonably quickly and efficiently to this available local energy minimum. In the simplified example shown in Figure 3, the interactions that stabilize the conformation of the nascent chain are also present in the native structure; in reality, this will probably depend on the topology of the native structure.

As even more of the nascent chain appears during translation, additional conformational space is added and additional portions of the funnel become accessible to the growing polypeptide chain. Eventually, synthesis of the polypeptide is completed. In the period when the chain is full length but has not been released from the ribosome, the chain has a vast number of conformations available and can access most, although not all, of the full folding funnel (Figure 3); some portions of the funnel remain inaccessible because they require the chain to be free from the conformational restrictions of the ribosome tether.

This gradual cotranslational 'exposure' to the folding funnel might explain why proteins that do not interact with molecular chaperones still fold to a much higher yield in the cell than in the test tube. By initiating the folding process from a defined trajectory rather than from all possible starting conformations, the chain is predisposed to fold by a prescribed route. This route might very well bypass vast segments of conformational space that are particularly prone to long-lived, aggregation-prone folding intermediates (Figure 3). Indeed, experimental measurements of the conformations of cotranslational folding intermediates indicate that some nascent chains adopt conformations that are distinct from the dominant conformations that are populated during *in vitro* refolding experiments [39,46,47].

It is important to note that the scheme shown in Figure 3 assumes that the polypeptide chain makes only native intramolecular contacts during folding and can build up these contacts vectorially (from the N to the C terminus). The cotranslational folding process of many proteins, however, has additional complications. For example, many protein structures have extensive contacts between amino acids that are distant from one another in the primary structure; for example, such contacts frequently occur in parallel β-sheet topologies and complex antiparallel β-strand topologies. For these proteins, nonnative contacts might develop cotranslationally because the N-terminal portions of the sequence interact nonnatively to form transiently stable intermediates until the C-terminal segments (or other subunit polypeptide chains) required for the native fold appear [46,48]. Presumably, folding to the native structure would require any early intermediates formed from the N-terminal sequence to be 'unraveled', representing an energy barrier in the cotranslational folding funnel. Notably, an implication of the cotranslational funnels is that the rate-limiting step for cotranslational folding might be distinct from that observed for refolding *in vitro*.

Conclusions and future directions

How does a double folding funnel affect our view of protein folding in the cell? Clearly, the hurdle for polypeptide chains lies not in 'skiing' down the folding funnel, but in avoiding the aggregation funnel. Short proteins that fold very quickly *in vitro* without intermediates [49–51] might have *in vivo* folding funnels that maintain more of the features of the corresponding *in vitro* refolding funnels, and therefore do not require any interactions with cell

components when folding *in vivo*. For most proteins, however, the double funnel diagram highlights the importance of early conformational arrangements, located near the tops of the funnels, in determining their folding fate. Some early conformations (and some polypeptide sequences) are expected to be more prone to aggregation than others, on the basis of fundamental features such as the clustering of hydrophobic amino acids [52] and the intrinsic conformational preferences of the denatured ensemble [53,54].

The features of the chaperone-mediated and cotranslational folding funnels lead to a suggestion that the populated intermediate conformations and alternative (parallel) folding pathways observed during refolding *in vitro* might not have a significant role in polypeptide chain folding *in vivo*. These intermediate conformations might not be significantly populated *in vivo*: they might be bypassed during cotranslational development of the folding funnel. Alternatively, intermediates that do accumulate *in vivo* might be quickly recognized by the cellular components (such as molecular chaperones) responsible for fine-tuning the maturation of the protein and for controlling the competing aggregation reaction. These mechanisms might act as guides to direct the polypeptide chains into the folding funnel or to block access to the aggregation funnel.

Applied to the folding funnel landscape, cotranslational folding and interactions with other cellular components might result in a radical reshaping of the conformational space explored by many polypeptide chains *in vivo*. Experiments designed to test these models might prove more valuable for understanding the success or failure of protein folding in the cell than exploring whether *in vitro* refolding intermediates are 'on-pathway' or 'off-pathway' for productive folding.

Acknowledgements

Thanks to Jonathan King, Ken Dill, Dan Gezelter, Brian Baker, Seth Brown and members of the Clark laboratory for fruitful discussions, numerous funnel doodling sessions, and/or critically reading the article. This work was supported by awards from the National Science Foundation, the American Heart Association, and the Clare Boothe Luce Program of the Henry Luce Foundation.

References

1 Mitraki, A. *et al*. (1987) Quasi-irreversibility in the unfolding–refolding transition of phosphoglycerate kinase induced by guanidine hydrochloride. *Eur. J. Biochem.* 163, 29–34
2 Harrington, W. and Rao, N.V. (1970) Collagen structure in solution. I. Kinetics of helix regeneration in single chain gelatins. *Biochemistry* 9, 3714–3724
3 Mitraki, A. and King, J. (1989) Protein folding intermediates and inclusion body formation. *Biotechnology* 7, 690–697
4 Wetzel, R. (1996) For protein misassembly, it's the 'I' decade. *Cell* 86, 699–702
5 Anfinsen, C.B. and Haber, E. (1961) Studies on the reduction and re-formation of protein disulphide bonds. *J. Biol. Chem.* 236, 1361–1363
6 Goldberger, R.F. *et al*. (1963) Acceleration of reactivation of reduced bovine pancreatic ribonuclease by a microsomal system from rat liver. *J. Biol. Chem.* 238, 628–635
7 Hartl, F.U. *et al*. (1994) Molecular chaperones in protein folding: the art of avoiding sticky situations. *Trends Biochem. Sci.* 19, 20–25
8 King, J. *et al*. (1996) Thermolabile folding intermediates: inclusion body precursors and chaperonin substrates. *FASEB J.* 10, 57–66
9 Raso, S.W. and King, J. (2000) Protein folding and human disease. In *Mechanisms of Protein Folding* (Pain, R.H. ed.), pp. 406–422, Oxford University Press
10 London, J. *et al*. (1974) Renaturation of *Escherichia coli* tryptophanase after exposure to 8 M urea. Evidence for the existence of nucleation centers. *Eur. J. Biochem.* 47, 409–415
11 Speed, M.A. *et al*. (1995) Multimeric intermediates in the pathway to the aggregated inclusion body state for P22 tailspike polypeptide chains. *Protein Sci.* 4, 900–908
12 Hulmes, D.J. (2002) Building collagen molecules, fibrils, and suprafibrillar structures. *J. Struct. Biol.* 137, 2–10
13 Lomas, D.A. *et al*. (1993) Effect of the Z mutation on the physical and inhibitory properties of α_1-antitrypsin. *Biochemistry* 32, 500–508
14 Dobson, C.M. (2001) The structural basis of protein folding and its links with human disease. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 356, 133–145
15 Tycko, R. (2004) Progress towards a molecular-level structural understanding of amyloid fibrils. *Curr. Opin. Struct. Biol.* 14, 96–103
16 Lashuel, H.A. *et al*. (2002) α-Synuclein, especially the Parkinson's disease-associated mutants, forms pore-like annular and tubular protofibrils. *J. Mol. Biol.* 322, 1089–1102
17 Dill, K.A. (1985) Theory for the folding and stability of globular proteins. *Biochemistry* 24, 1501–1509
18 Bryngelson, J.D. and Wolynes, P.G. (1987) Spin glasses and the statistical mechanics of protein folding. *Proc. Natl. Acad. Sci. U. S. A.* 84, 7524–7528
19 Betts, S.D. *et al*. (1997) Mutational effects on inclusion body formation. *Adv. Protein Chem.* 50, 243–264
20 Marston, F.A.O. (1986) The purification of eukaryotic polypeptides synthesized in *Escherichia coli*. *Biochem. J.* 240, 1–12
21 Istrail, S. *et al*. (1999) Lattice simulations of aggregation funnels for protein folding. *J. Comput. Biol.* 6, 143–162
22 Dima, R.I. and Thirumalai, D. (2002) Exploring protein aggregation and self-propagation using lattice models: phase diagram and kinetics. *Protein Sci.* 11, 1036–1049
23 Smith, A.V. and Hall, C.K. (2001) Protein refolding versus aggregation: computer simulations on an intermediate-resolution protein model. *J. Mol. Biol.* 312, 187–202
24 Liu, Y. and Eisenberg, D. (2002) 3D domain swapping: as domains continue to swap. *Protein Sci.* 11, 1285–1299
25 Dill, K.A. and Chan, H.S. (1997) From Levinthal to pathways to funnels. *Nat. Struct. Biol.* 4, 10–19
26 Locker, C.R. and Hernandez, R. (2001) A minimalist model protein with multiple folding funnels. *Proc. Natl. Acad. Sci. U. S. A.* 98, 9074–9079
27 Massi, F. and Straub, J.E. (2001) Energy landscape theory for Alzheimer's amyloid β-peptide fibril elongation. *Proteins Struct. Funct. Genet.* 42, 217–229
28 Mitraki, A. *et al*. (1991) Global suppression of protein folding defects and inclusion body formation. *Science* 253, 54–58
29 Wigley, W.C. *et al*. (2002) A protein sequence that can encode native structure by disfavoring alternate conformations. *Nat. Struct. Biol.* 9, 381–388
30 Richardson, J.S. and Richardson, D.C. (2002) Natural β-sheet proteins use negative design to avoid edge-to-edge aggregation. *Proc. Natl. Acad. Sci. U. S. A.* 99, 2754–2759
31 van der Vies, S.M. *et al*. (1992) Conformational states of ribulose-bisphosphate carboxylase and their interaction with chaperonin 60. *Biochemistry* 31, 3635–3644
32 Ellis, R.J. and Hartl, F.U. (1996) Protein folding in the cell: competing models of chaperonin function. *FASEB J.* 10, 20–26
33 Baumketner, A. *et al*. (2003) Effects of confinement in chaperonin assisted protein folding: rate enhancement by decreasing the roughness of the folding energy landscape. *J. Mol. Biol.* 332, 701–713
34 Ellis, R.J. (2001) Macromolecular crowding: obvious but underappreciated. *Trends Biochem. Sci.* 26, 597–604
35 Jaswal, S.S. *et al*. (2002) Energetic landscape of α-lytic protease optimizes longevity through kinetic stability. *Nature* 415, 343–346
36 Hartl, F.U. and Hayer-Hartl, M. (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* 295, 1852–1858
37 Bukau, B. *et al*. (2000) Getting newly synthesized proteins into shape. *Cell* 101, 119–122

38 Frydman, J. (2001) Folding of newly translated proteins *in vivo*: the role of molecular chaperones. *Annu. Rev. Biochem.* 70, 603–647
39 Fedorov, A.N. and Baldwin, T.O. (1999) Process of biosynthetic protein folding determines the rapid formation of native structure. *J. Mol. Biol.* 294, 579–586
40 Seckler, R. *et al*. (1989) Reconstitution of the thermostable trimeric phage P22 tailspike protein from denatured chains *in vitro*. *J. Biol. Chem.* 264, 11750–11753
41 Komar, A.A. *et al*. (1997) Cotranslational folding of globin. *J. Biol. Chem.* 272, 10646–10651
42 Nicola, A.V. *et al*. (1999) Co-translational folding of an alphavirus capsid protein in the cytosol of living cells. *Nat. Cell Biol.* 1, 341–345
43 Malkin, L.I. and Rich, A. (1967) Partial resistance of nascent polypeptide chains to proteolytic digestion due to ribosomal shielding. *J. Mol. Biol.* 26, 329–346
44 Woolhead, C.A. *et al*. (2004) Nascent membrane and secretory proteins differ in FRET-detected folding far inside the ribosome and in their exposure to ribosomal proteins. *Cell* 116, 725–736
45 Bernabeu, C. and Lake, J.A. (1982) Nascent polypeptide chains emerge from the exit domain of the large ribosomal subunit: immune mapping of the nascent chain. *Proc. Natl. Acad. Sci. U. S. A.* 79, 3111–3115
46 Clark, P.L. and King, J. (2001) A newly synthesized, ribosome-bound polypeptide chain adopts conformations dissimilar from early *in vitro* refolding intermediates. *J. Biol. Chem.* 276, 25411–25420
47 Frydman, J. *et al*. (1999) Co-translational domain folding as the structural basis for the rapid *de novo* folding of firefly luciferase. *Nat. Struct. Biol.* 6, 697–705
48 Chow, C.C. *et al*. (2003) Chain length dependence of apomyoglobin folding: structural evolution from misfolded sheets to native helices. *Biochemistry* 42, 7090–7099
49 Jackson, S.E. and Fersht, A.R. (1991) Folding of chymotrypsin inhibitor 2.1. Evidence for a two-state transition. *Biochemistry* 30, 10428–10433
50 Schindler, T. *et al*. (1995) Extremely rapid protein folding in the absence of intermediates. *Nat. Struct. Biol.* 2, 663–673
51 Sosnick, T.R. *et al*. (1996) Molecular collapse: the rate-limiting step in two-state cytochrome *c* folding. *Proteins Struct. Funct. Genet.* 24, 413–426
52 Schwartz, R. *et al*. (2001) Frequencies of amino acid strings in globular protein sequences indicate suppression of blocks of consecutive hydrophobic residues. *Protein Sci.* 10, 1023–1031
53 Chiti, F. *et al*. (2002) Kinetic partitioning of protein folding and aggregation. *Nat. Struct. Biol.* 9, 137–143
54 Klein-Seetharaman, J. *et al*. (2002) Long-range interactions within a nonnative protein. *Science* 295, 1719–1722
55 Onuchic, J.N. *et al*. (1997) Theory of protein folding: the energy landscape perspective. *Annu. Rev. Phys. Chem.* 48, 545–600