Conformational control through translocational regulation: a new view of secretory and membrane protein folding

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Summary

We suggest a new view of secretory and membrane protein folding that emphasizes the role of pathways of biogenesis in generating functional and conformational heterogeneity. In this view, heterogeneity results from action of accessory factors either directly binding specific sequences of the nascent chain, or indirectly, changing the environment in which a particular domain is synthesized. Entrained by signaling pathways, these variables create a combinatorial set of necessary-but-not-sufficient conditions that enhance synthesis and folding of particular alternate, functional, conformational forms. We therefore propose that protein conformation is productively regulated by the cell during translocation across the endoplasmic reticulum (ER), a concept that may account for currently poorly understood aspects of physiological function, natural selection, and disease pathogenesis. ***BioEssays*** **24:741–748, 2002.**

Introduction

Protein biogenesis in the secretory pathway involves several processes that overlap temporally with polypeptide chain synthesis (see Fig. 1). First, the nascent chain must be targeted[(1)] correctly to the membrane of the endoplasmic reticulum (ER). Then, its translocation to the lumen of the ER must be initiated.[(2)] Folding of the chain has to begin early in translocation.[(3)] During and immediately after translocation, post-translational modifications occur,[(4)] and decisions need to be made regarding degradation of undesired chains, including translocation back to the cytoplasm and degradation by the proteasome.[(5)] A great deal is known about some features of targeting and translocation per se.[(1,2)] But little is known about how these processes are coordinated with the aforementioned events, or how any of these events are regulated during the biogenesis of complex secretory and membrane proteins.

For the purposes of this discussion, a protein may be categorized as "simple" if it is made, translocated, folded and exported efficiently, undergoing few biologically relevant interactions until after it has been secreted from the synthesizing cell. "Complex" proteins are those in which there is a structural, functional or regulatory basis for variation on the themes of the above events. For example, some secretory proteins have domains that carry lipids or other substances, and their loading with cargo must occur during or after synthesis but before secretion.[(6)] Some complex proteins associate with other polypeptides,[(7)] localize to specific compartments within the secretory pathway,[(8)] or seem to serve multiple functions in the cell.[(9)] Complex proteins are also involved in disease in ways that are not easily understood in terms of simple excess or deficiency of a particular protein product.[(10)]

Of the steps in protein biogenesis, translocation across the ER is "special" because it occurs while the chain is nascent, a time when commitment to a particular final folded state has not yet been made. Teleologically speaking, this would be an ideal time for the cell to regulate the pathway of any associated process. Of the processes that occur contemporaneously with translocation, perhaps the most profound is protein folding, because it is a crucial step in the decoding of the information in the genome. A misfolded protein may be as bad—or worse—than not having the protein at all. Further, if proteins have multiple folded states with distinct functions, the precise pathway of folding and its regulation will determine which function is actually expressed, and to what extent.

Both the kinetics and thermodynamics of folding are likely to vary with the environment in which they occur. During translocation, the growing chain resides in a channel, termed the translocon, comprising the space from the ribosome to the ER lumen. The translocon however is more than just a channel

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DOI 10.1002/bies.10130
Published online in Wiley InterScience (www.interscience.wiley.com).

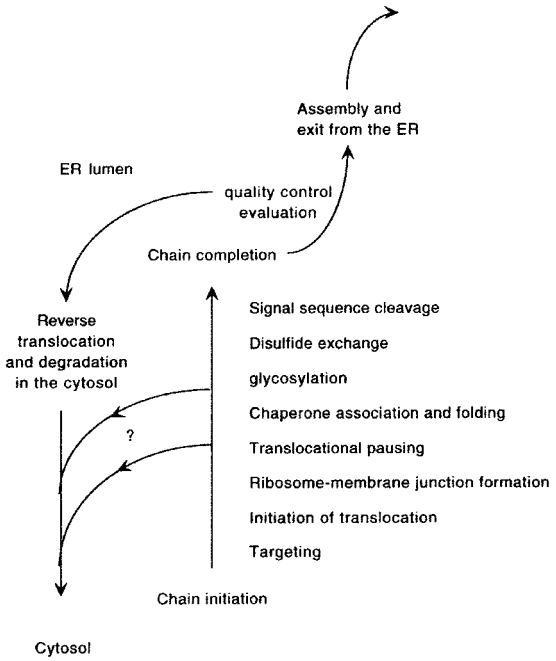

Figure 1. The conventional view of early events of protein biogenesis. Between initiation of protein synthesis and export from the ER, nascent secretory and membrane proteins are subjected to a complex assembly line of modifications in space and time. Each of these modifications are due to protein–protein interactions, and to the extent that any given modification does not occur on all chains of the population, an opportunity is created to alter the pathway of folding taken by a subpopulation of chains. Note that the events indicated are likely to be only a small subset of the actual or potential events occuring during translocation, do not necessarily happen in the particular sequence indicated, and may vary and overlap in time with one another, from protein to protein.

across the membrane. It is a specialized, protected space, which possesses both the characteristics of an aqueous channel[11] and some of the hydrophobic character of the lipid bilayer it traverses.[12] It is also a crossroads from which, over time, the nascent chain potentially is accessible to the reducing environment of the cytosol at one end, the oxidizing environment of the ER lumen at the other, and the distinctive proteins in each compartment. Recent evidence suggests that some nascent substrates avail themselves of the full range of potential for changing the environment in which the chain grows which has consequences that include differences in folding.[13–15]

Principles of translocation across the ER membrane

Several features of protein translocation across the ER membrane are relevant for protein folding. First, translocation is a receptor-mediated process,[16] albeit a non-classical one. The signal sequence is a discrete stretch of amino acids within the nascent chain that constitutes the ligand. Binding of this ligand to "receptors" causes targeting to the ER membrane, and sets in motion a cascade of protein–protein interactions resulting in translocation of the "passenger" polypeptide, which flanks the signal sequence. The receptors for targeting, translocation and associated events are proteins or protein complexes in the cytosol, the ER membrane and the ER lumen. Their function is distinctive in that many of their interactions with nascent chain ligands are likely to be of relatively low affinity, driven primarily by proximity within the translocon (high effective concentration). Furthermore, unlike classical receptors (e.g. for polypeptide hormones), these interactions are intrinsically transient; the process never comes to equilibrium because there is continued input of energy, due to nascent chain elongation and the environment surrounding the ligand varies as the chain grows and folds.

Second, the translocon is fundamentally an aqueous space.[11,17] However its internal character is not necessarily fixed but may vary (in terms of dimension and extent of hydrophobic surface exposed within the channel) with the substrate being translocated.[18] This flexibility may allow partially folded domains to be accommodated within, and moved through, the translocon. Likewise, multiple transmembrane regions of integral membrane proteins may be oriented, positioned, folded, and assembled within the translocon prior to integration into the bilayer.[19,20] The translocation channel can be thought of as a conveyor belt in an assembly line, which allows work to be performed on the chain during its movement from ribosome to ER lumen. This work may include alterations such as post-translational modifications, protein–protein interactions, and both spontaneous and energy-driven features of protein folding.

Third, there are important variations on the theme of simple secretory protein translocation. Prominent among these is the biogenesis of integral membrane proteins, most of which seem to depend on additional intra- and inter-molecular interactions involving the nascent chain, the translocon, and/or the lipid bilayer. For membrane proteins that target to the ER using an N-terminal signal sequence, a stop transfer sequence typically emerges as part of the subsequent nascent chain to serve as a ligand to terminate translocation.[21–24] Alternatively, the signal and stop transfer sequence functions can be combined in a so-called signal-anchor sequence that promotes the translocation of one of its flanking domains.[25] In the case of multi-spanning membrane proteins, various topogenic sequences have been identified.[26] Their actions are complex and often not readily understood in terms of sequential, disparate, receptor–ligand interactions,[27] although simple signal and stop transfer sequences can be used to build transmembrane proteins of predictable orientation.[28,29] Yet other sequences within the nascent chain appear to serve functions that are distinct from translocation per se. For example, pause

transfer sequences can cause an opening of the normally closed ribosome–membrane junction, thereby allowing a defined region of the nascent chain access to the cytosol, a distinctly different environment from the translocon and ER lumen.[30,31] The existence of these variations implies that the translocon is both extremely versatile and complex. The core components have been defined,[32] but many accessory components have still to be better understood.[33] The combinatorial possibilities for interactions among ligands, receptors, and compartments as a function of time and chain folding are immense.

Principles of protein folding

A fundamental dogma of modern biology is that primary structure determines secondary structure, which, together with appropriate post-translational modifications such as disulfide bond formation, determines the tertiary (and quaternary) protein structures.[34] This organization, from primary structure → secondary structure → tertiary structure, constitutes a "first order" organizing principle for protein folding. Implicit in the term "structure" is the notion of a unique, stable entity; but protein structure is a statistical concept. Native protein conformations are energetically preferred relative to unfolded, denatured forms of the same chains; but the energetic preference usually is modest (~10 kCal/mole). Proteins are dynamic, fluctuating entities, and even "stable"proteins will unfold transiently, to some degree (at any moment, $\sim 10^{-7}$ of the proteins will be unfolded). In addition to including the importance of conformational fluctuations, schemes for protein folding need to be enhanced to account for the fact that in vivo folding occurs faster, and in a much more crowded environment, than usually is the case for purified proteins in solution.

Molecular chaperones are proteins that enhance the fidelity of folding by preventing inappropriate (intermolecular) interactions, thereby facilitating the process of achieving a correct (final) folded state. Chaperones account in part for why folding in vivo occurs much more efficiently than in vitro.[35] They also may prevent transient partial unfolding from leading to disaster –in the sense that these partially unfolded proteins will not accumulate and aggregate to cause disease. Chaperone-assisted folding can be thought of as "second order" folding because it occurs in a specialized, confined space and depends on ATP hydrolysis—features that introduce new levels of complexity. Applying this notion to protein translocation, the ribosome and the core translocon (connected via the ribosome–membrane junction) serve together as a molecular chaperone for folding the nascent chains.[58] Not only does the translocon recognize and bind sequences in the nascent chain, but it also controls the environment surrounding the polypeptide. Given the extensive literature describing the effects of different solvents on the secondary structure of polypeptide chains,[36] the changing environment that is experienced by the nascent chain may be crucial for proper chain folding. Similarly, polar–non-polar interfaces can also organize the secondary structure of peptide ~20 residues in length,[37] effects which most likely will be reflected in alterations of tertiary structures of the larger protein.

The channel-forming peptide antibiotic gramicidin A (gA)[38] provides a particularly striking example of such environment-dependent folding. In organic solvents, gA can exist in at least seven different, interconverting conformations.[39] All of these differ from the predominant channel conformation, which is a single-stranded, bilayer-spanning dimer.[40] In lipid bilayers, the channel conformation varies as a function of the discordance between channel length and the thickness of the bilayer hydrophobic core.[41] A striking parallel can be drawn between the ability of different solvents to affect protein folding and the ability of machinery within the cell to regulate the environment in which folding occurs, as discussed above.

In addition to the possible role of the environment, it is important to recognize that folding may be initiated in different parts of the chain at (almost) the same time. If this is the case, it becomes necessary to leave behind the linear kinetic schemes traditionally used to describe protein folding and rather emphasize the energy landscapes and folding "funnels" that describe the process.[42] If the folding funnel has fairly smooth walls and a single, well-defined free energy minimum, the protein-folding problem would be relatively simple, even if it was far from solved. By analogy with multiparameter, nonlinear fitting problems, however, the sides of the funnel will likely be "bumpy", with pronounced "ravines"—and the bottom of the funnel will be sufficiently flat and irregular that no single, well-defined energy minimum exists. As long as the major energy minima differ by less than 1.4 kCal/mole, or so, the relative concentrations of the different conformers will differ by less than tenfold. For comparison, the strength of a hydrogen bond is usually assumed to be ~3 kCal/mole, so there is plenty of energy available to allow the proteins to explore the folding landscape.

An important implication of this line of thinking is that the folded state need not be the global minimum free energy state, which means that the "final" folded state may be determined by both kinetic and equilibrium factors.[43,44] The chain may funnel toward a global energy minimum but, if there are significant bumps and ravines in the folding funnel, the protein could become "trapped" in one or more secondary minima, each with their characteristic conformation. The biologically active conformation of plasminogen activator inhibitor 1 is, for example, not the lowest free energy state.[45]

The notion of kinetic control of folding becomes particularly intriguing when noting that one can alter the conformational preference of a protein by altering less than 50% of the amino acids in the sequence.[46] If kinetic control of folding is important, the conformation would depend on which sequence segment forms the initial nucleus of native structure and the

choice of ravine that was followed when the chain hits a bump in the funnel. The central issue thus becomes, how does the cell select one versus another possible relative energy minimum in the protein-folding landscape? Choosing the one time in its life that a protein is nascent contributes importantly to solving this problem because no commitment has yet been made. Exposure of the chains to different environments also contributes to the solution to this problem. Each of these issues is relevant to protein translocation. First, the translocation process involves a series of regulated exposures to different environments, allowing partially folded segments of chain to explore a larger part of the local energy landscape. Second, the transient, non-equilibrium interactions between ligands within the nascent chain and various receptors of translocation may influence the way a substrate negotiates the various bumps and ravines of the folding funnel.

Interrelationship of translocation and folding

Because translocation overlaps with protein folding, one would expect that, in the course of evolution, these two processes have influenced one another. Folding pathways may have been modified to accommodate the needs of translocation; translocation pathways may have been modified to accommodate the needs of folding. A growing body of literature provides support for both of these possibilities.

The most dramatic example to date of translocational regulation, with implications for folding, is seen in the biogenesis of the prion protein (PrP). In the case of PrP, a homogeneous population of nascent chains results in three topological forms.[47] One of them, ^{sec}PrP, appears to be fully translocated (secreted) across the ER membrane and tethered by a C-terminal glycolipid anchor; this is the form observed in normal brain. Although the function of ^{sec}PrP is unknown, it seems likely, by analogy to other glycolipid anchored proteins, to have signaling functions in the nervous system.[48] A recently demonstrated anti-apoptotic function appears consistent with this role.[49] The other two forms of PrP span the membrane once in opposite orientations, with a membrane-spanning stretch at approximately amino acids 112–130. One of these forms, ^{Ctm}PrP, which has its C terminal domain translocated to the ER lumen, triggers spontaneous neurodegeneration when overexpressed.[47] Furthermore, in infectious prion disease, ^{Ctm}PrP appears to be induced just prior to onset of clinical signs, suggesting that it initiates a final common pathway to neurodegeneration.[50] Other studies implicate an as yet unknown glycoprotein of the ER membrane as a translocation accessory factor (TrAF) that "protects" the normal brain from expression of ^{Ctm}PrP by directing nascent PrP chains to the pathway leading to ^{Sec}PrP.[33]

The mechanism by which nascent PrP chains are allocated among the topological forms is complex.[13–15] The PrP signal sequence itself may play a role by establishing at least two populations of nascent chains: some with an open ribosome–membrane junction, others with a closed ribosome–membrane junction. Thus, the signal sequence can determine the environment encountered by the emerging N-terminal domain of PrP. Only the chains exposed to the cytosolic environment have the potential to become ^{Ctm}PrP. While necessary, however an open ribosome–membrane junction is not sufficient to make ^{Ctm}PrP, as additional protein–protein interactions appear to determine the final outcome. Mutations that prevent the transmembrane domain from directing ^{Ctm}PrP formation result in these chains being redirected to the cytosol, where they most likely are degraded.

The distinction between ^{Sec}PrP and ^{Ctm}PrP are usually made on topological grounds. However, these two polypeptides of identical sequence also differ in their conformation. This was demonstrated by their differential sensitivity to limited protease digestion in non-denaturing detergent solutions.[47] Thus, translocational regulation appears to be a means of generating multiple forms of PrP that differ in both conformation and function. The machinery (i.e. a TrAF) that directs nascent PrP chains to make ^{Sec}PrP rather than ^{Ctm}PrP, may itself be regulated, based on the ability of scrapie infection to increase the amount of ^{Ctm}PrP detectable in brain.[50]

Although PrP is currently the best example of translocational regulation, there is evidence for similar principles being utilized by a broader set of proteins.[51,52] Together, these observations lead to a new principle: a protein's conformation is determined not just by its primary amino acid sequence, but also by proteins such as TrAF, that influence which of two or more different functional conformational outcomes actually occur or predominate.

A general hypothesis of translocational regulation

Taking PrP as our starting point, we propose that complex secretory or integral membrane proteins can have multiple distinct functional folded states. The different folded states become manifest by a combinatorial series of interactions between ligands within the nascent chain and receptors at the translocon, in an array of possible environments (e.g. cytosol, membrane, lumen). The rates of generation versus degradation of the various conformations determine which, and how many, of the possible final outcomes for a particular substrate are expressed at any given time. These processes are integrated by signaling pathways from the cell surface and elsewhere, which coordinate protein biogenesis with both the specific immediate needs[53] and the global program of the cell.[54] Consequences of a change in transient or final folded state could include changes in the mix of modifications on a given protein chain (e.g. such as phosphorylation or glycosylation), or a change in regulation or function of a particular subfraction of chains representing the alternate conformational form.

According to this view secretory and integral membrane nascent chains proceed down more than one folding funnel (or take different paths to different ends within a single, complex folding funnel). This is accomplished through the interaction of discrete sequences in the nascent chain with TrAFs, or with the environments that TrAFs make accessible. Pathway choice could also be biased by the continued supply of energy, as the nascent chain is being synthesized or modified. Different TrAFs, localized to different compartments (cytosol, membrane, ER lumen), that can act on different chains or different subsets of folding states of a given chain also contributes to the selection of one functional folded form versus another.[33] This can be thought of as "third-order" complexity in protein folding. Thus, any given secretory or integral membrane protein could exist in multiple conformations, each with a distinctive functional signature, with the mix of different conformations changing in response to changed physiological (or pathological) circumstances. This notion differs from the classical concept of molecular chaperones in that the consequence of lack of TrAF action is not an accumulation of misfolded, non-functional copies of the protein, as would occur without molecular chaperone action. Rather, in the absence of TrAFs, subpopulations of chains of distinctive structure (conformers), with a different function or regulation, would be lost.

Finally, the translocon and its components including TrAFs, that bias the choice among possible alternative folding funnels could themselves be subject to regulation by intracellular signaling, thereby providing a "fourth order" of complexity to protein folding (see Table 1). The signaling pathways in turn are responsive to the needs of the cells, which creates the "interface" to a host of other mechanisms of metabolic regulation.[53,54] The recent in vivo and in vitro observations regarding prion protein biogenesis suggest strongly that such mechanisms of regulation must exist.[13–15,33,47,50,52] Fig. 2 summarizes a working model of translocational regulation.

Predictions, implications, and future directions

The most-immediate prediction of this model is that it should be possible to engineer into a model protein a reporter of folding (e.g. a glycosylation site), and demonstrate that altering the pathway of biogenesis (e.g. by swapping or mutagenesis of signal sequences) results in an altered conformation (e.g. altered pattern of glycosylation). Furthermore, the signal sequence alterations that direct the altered glycosylation should also alter the organization of the translocon in a manner that is not dependent on glycosylation per se (i.e occurs even when glycosylation is blocked). This has recently been demonstrated for a model secretory protein.[52]

Several implications follow directly from this view of translocational regulation and folding. Most immediately, the information content of the genome would be substantially increased from what is currently believed, due to the ability of any given coding region to be expressed in many different conformations, each with a different function. The actual increase would depend on presently indeterminate factors; but it could be mind boggling. Regulation at the folding step could account in large part for the mechanisms by which the diversity and nuances of higher metazoan biology can be generated from a mere 30,000 (or so) functional genes encoding simple proteins. It may not be a coincidence that the brain[47] and the immune system,[55] perhaps the most complex systems of metazoan biology in terms of information content and processing, are where these novel modes of regulation of gene expression have been first detected.

The notion of translocational regulation also suggests a new mechanism for cellular adaptation to a changing environment. By merely modifying (e.g., via phosphorylation), inactivating or degrading individual TrAFs in response to environmental stimuli, the program of the cell could be rapidly and radically changed. For example, the "default" folding pathway for PrP in the absence of TrAF action is ^{Ctm}PrP, the topological isoform that causes neurodegeneration. Thus (one of) the physiological function of PrP may be to serve as one (of perhaps several) cellular baromometers of environment that trigger neurodegeneration through its own translocational regulation.

Translocational regulation also provides a means by which the tempo of evolution could be enhanced because the "raw material" for selection, represented by different conformations, would be plentiful and available. Furthermore, new

Table 1. Organizing principles for the levels of complexity in protein folding

Degrees of complexity	Paradigm	Distinguishing features
1st order	1° structure > 2° structure > 3° structure	Spontaneous, thermodynamically driven process
2nd order	Molecular chaperones	Proteins that indirectly facilitate "correct" folding by binding unfolded proteins and preventing inappropriate folding. ATP-dependent; Compartment-specific;
3rd order	Translocation acessory factors (TrAFs)	Proteins that direct nascent secretory and membrane proteins to alternative pathways of topogenesis or folding
4th order	Signaling pathways	Entrained by physiological variables to achieve homeostasis

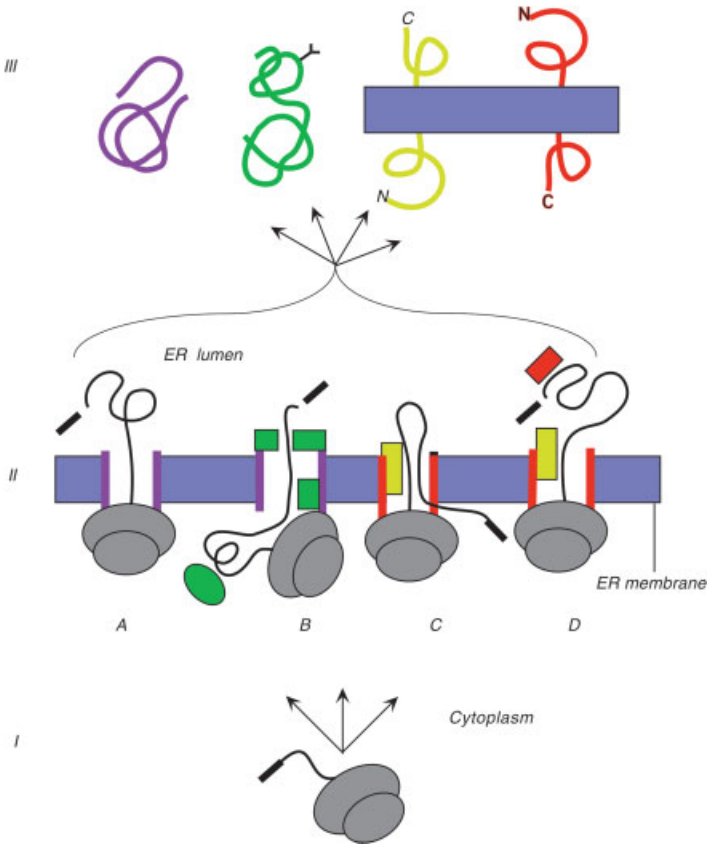

Figure 2. How translocational regulation can lead to conformational heterogeneity. Events proceed temporally from bottom to top. Indicated in Roman numerals to the left are the endpoints of three stages of protein biogenesis at the ER: (I) the earliest events including targeting to the ER membrane; (II) the events constituting the pathway of translocation; and (III) the final folded conformers. Initially, the population of nascent chains is homogeneous (see stage I). Translocational regulation, of which four forms are indicated as A–D in stage II, provides a means by which the initially homogeneous population is subdivided into heterogeneous subpopulations. As a result, the final folded forms are different either in conformation per se (e.g. A versus B), or in modifications (e.g. A versus B), or in topology (e.g. C versus D), as hypothesized here. Molecular chaperones are indicated by colored ovals, while TrAFs are depicted as colored rectangles. **A:** The translocon lacks TrAFs resulting in a distinctive skew in the conformer mix towards that depicted as purple. **B:** An open ribosome–membrane junction , forces the nascent chain to initiate folding in a reducing environment, perhaps in association with molecular chaperones or machinery for post-translational modifications. These distinctive protein–protein interactions culminate in predominance of a conformer depicted as green. Note that the lumenal gate of the translocon is closed, while that on the cytoplasmic side that makes up the ribosome–membrane junction, is open. **C:** The translocon, indicated in red to represent either a different pathway of folding taken within the translocon or a different organization of translocon components compared to the purple translocons in A and B, including a TrAF (in yellow) to direct transmembrane topology. **D:** The action of additional TrAFs can result in a change in protein topology as well as conformation. The examples of translocational regulation indicated here could apply to different subpopulations of an initially homogeneous population of nascent chains as a consequence of regulatory events, for example, functional alteration of a signal sequence upon binding of a protein. Alternatively these conformational and topological outcomes could represent the different fates achieved by different populations of chains bearing different classes of signal sequences.

TrAF–substrate interactions could evolve, with the consequent generation of even more conformations, in response to selection pressure. Over time, the percentage of chains in conformations that proved useful under given circumstances would be amplified, and those that were deleterious would be suppressed. More importantly, by having the level of expression of specific forms contingent on the state of activation of various signaling pathway, cells could evolve new developmental and functional programs from pre-existing ones by means of conformational regulation at the translocational level. It would be necessary to screen this flow of information through the quality control machinery of the ER lumen—including degradative pathways involving proteasome, redox status, etc.—so that the most deleterious changes would be eliminated. Changes of potential value could be rescued and amplified in the specific circumstances in which they conferred

selective advantage. In any event, the energy costs of regulation at this level, which requires no change in transcription, splicing, mRNA transport or even translation, may be substantially less than the alternatives.

At present, the major limitation to testing these and other hypotheses of conformational control is a lack of tools to recognize the heterogeneity of functional protein conformations. But for the fortuitous coincidence that, in PrP, conformational heterogeneity was expressed as topological heterogeneity, it might not yet have been detected. Indeed, the consequences of translocational regulation for other substrates remains unknown, despite the fact that evidence for their transient exposure to the cytosol during translocation has been abundantly clear for years.[56] In the light of the findings on PrP, it seems likely that the consequences include alterations in protein folding, but this remains to be fully demonstrated.

Better tools (and more of them) are needed if this problem is to be addressed experimentally. For example, panels of conformation-specific monoclonal antibodies would allow relative reactivities of subpopulations of newly synthesized proteins to be scored, and thereby define conformational differences. This approach should allow a catalogue of the conformational states utilized by a given protein in health and during the progression to disease. However, a comprehensive exploration of these concepts will require general methods for separating otherwise identical molecules based solely on subtle conformational differences, perhaps involving novel types of chromatography. Finally, animal models such as transgenic mice will need to be manipulated in ways that allow exploration of the functional roles of specific protein conformations. This latter approach is already underway in neurodegenerative diseases.[57]

This review has been an attempt to hybridize new concepts emerging from the fields of protein biogenesis and protein folding leading to the appreciation of two new orders of complexity to the protein-folding problem. The challenge for the future appears to be a hybrid of Pasteur's aphorism "chance favors the prepared mind" with the observation attributed to Dewey that "the givens of experience are not given, but rather are taken, with great difficulty". A lot of painstaking and attentive experimental work will be necessary to determine whether the concepts presented here can be generalized to many proteins or apply only to a specialized few.

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