Protein Folding and Modification in the Mammalian Endoplasmic Reticulum

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Abstract

Analysis of the human genome reveals that approximately a third of all open reading frames code for proteins that enter the endoplasmic reticulum (ER), demonstrating the importance of this organelle for global protein maturation. The path taken by a polypeptide through the secretory pathway starts with its translocation across or into the ER membrane. It then must fold and be modified correctly in the ER before being transported via the Golgi apparatus to the cell surface or another destination. Being physically segregated from the cytosol means that the ER lumen has a distinct folding environment. It contains much of the machinery for fulfilling the task of protein production, including complex pathways for folding, assembly, modification, quality control, and recycling. Importantly, the compartmentalization means that several modifications that do not occur in the cytosol, such as glycosylation and extensive disulfide bond formation, can occur to secreted proteins to enhance their stability before their exposure to the extracellular milieu. How these various machineries interact during the normal pathway of folding and protein secretion is the subject of this review.

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INTRODUCTION

The endoplasmic reticulum (ER) is the entry point for proteins to the secretory pathway (1). It is thought to have evolved by invagination of the plasma membrane. The result is a reticular membranous structure, which is contiguous with the nuclear membrane. As a consequence of its evolutionary origin, the internal volume or lumen of the ER is topologically equivalent to the outside of the cell. Proteins on their road to secretion in eukaryotic cells enter this intracellular organelle rather than being secreted directly across the plasma membrane as occurs in most prokaryotes. The development of this staging post in protein secretion has allowed eukaryotes to evolve a host of posttranslational modifications, which can take place within an intracellular compartment that is quite distinct from the cytosol, both in terms of protein and solute composition. Through the activities it contains, the ER also provides a checkpoint for secretion, providing a level of quality control on protein export, which in general only allows secretion of proteins that are folded and modified correctly.

The journey taken by a protein entering the mammalian ER starts with recognition of a signal sequence, typically but not exclusively found at the N terminus of the protein. This signal sequence is recognized by signal recognition particle (SRP) (2) with the resulting ribosome/nascent chain/SRP complex binding to the ER membrane via the SRP receptor. The ribosome/nascent chain complex then is directed toward a proteinaceous pore in the membrane called the Sec61 translocon, which allows the translocation of the growing polypeptide chain across the membrane and into the ER. The rapid targeting of the nascent polypeptide chain to the ER ensures that translocation occurs

cotranslationally, meaning that the folding of the polypeptide chain will take place within the lumen of the ER. Transmembrane proteins with large cytosolic domains are exceptions to a strict ER localization of folding, as they will need to fold in two additional environments, the membrane and cytosol. The exposure of a folding chain to a variety of ER lumen enzymes that catalyze posttranslational modifications of the amino acid side chain will result in a temporal relationship between the folding and modification of the polypeptide. It is therefore important to realize that protein folding may well influence posttranslational modification and that posttranslational modification can also influence folding.

Once fully translocated into the ER, the polypeptide will complete its folding and if part of a multisubunit complex will then assemble into its native oligomeric structure. Further posttranslational modifications may take place during this process. This period of maturation precedes the concentration of cargo protein into transport vesicles, which bud off from the ER prior to fusion with compartments such as the Golgi complex further down the secretory pathway. Proteins that fail to attain their native structure are retained in the ER, where they may be subsequently targeted for retrotranslocation back into the cytosol, and become ubiquitinated and degraded by the proteasome. Hence, the ER plays crucial roles in protein maturation, quality control, and recycling to ensure that only fully folded protein is delivered to its site of action.

Many recent reviews cover various aspects of the ER, including protein translocation (3), protein trafficking (4), and ER-associated protein degradation (5). In this review, we cover the process of protein folding that takes place in the mammalian ER with a particular reference to the various posttranslational modifications that either influence or are influenced by the collapse of the polypeptide chain. We also consider how the cell recognizes proteins that have not attained their native structure and how these proteins are retained within the ER.

PROTEIN FOLDING IN THE CELL AND IN ISOLATION

For decades before protein folding became amenable to studies in a biological context, the process had been studied in the test tube. A protein in isolation contains all information required for the three-dimensional structure and hence can be refolded in isolation (6). This folding code to date has not been cracked. Although attempts to predict a protein's fold from its sequence and to design novel proteins are increasingly successful (7), solved structures still are needed to feed most algorithms used. Until ~20 years ago, the in vivo and in vitro folding communities led separate lives and largely ignored each other. Simple reasons were the absence of overlap in techniques and conditions used, and proteins studied. Whereas in a cell complex multidomain proteins studded with posttranslational modifications fold with much success in warm, crowded climates, proteins in isolation do best in cool, diluted conditions and when they are small and relatively simple. A major difference that cannot be overcome easily is the sequence of events: Folding of isolated proteins starts with the complete protein, whereas in a cell, folding starts during synthesis, from the N to C terminus. This issue of cotranslational, vectorial folding has been a major impediment for biologists to accept in vitro refolding data as relevant. The simple facts are, however, that biophysical principles do apply in a cell and that chaperones and folding enzymes slow down folding in vivo to such an extent that the majority of folding may happen after synthesis of a protein. Essential for all comparisons is that the end points of folding in vivo and in vitro are the same and that hence the primary amino acid sequence encodes protein structure.

Following over half a century of studies, the driving forces behind protein folding have been determined, and models have been postulated for folding of a protein. With proteins being synthesized (and refolded in vitro) in an aqueous milieu, a strong general drive stems from the burial of hydrophobic side chains involving van der Waals interactions, called the

Hsp: heat shock protein

hydrophobic collapse (8). Hydrogen bonding and electrostatic interactions complete the noncovalent picture. Local interactions initially are favored over distant interactions, and nuclei of native structures may start to form early on as well (7). Every protein folds as determined by its sequence and therefore relatively uniquely, but each folding study has yielded information that fits into a global concept of protein folding and allows predictions for other proteins. Unifying models are being proposed and tested; a recent one described by Fersht (9), the nucleation condensation model of protein folding, pairs formation of a nucleus of native structure with condensation of the remainder of the protein around this nucleus, from which folding then proceeds to the native, properly folded state. Although these models are designed to explain folding of proteins in isolation, the folding process in intact cells shows features of these models, and therefore, we should not hesitate to apply them in vivo.

Crossing Over Between In Vivo and In Vitro Folding

The best test for similarity of protein folding in vivo and in isolation is to compare the folding of the same protein. This is a challenge because in vitro studies work best with smaller simpler proteins, whereas most mammalian proteins studied in vivo are large, multidomain, and complex, and undergo many posttranslational modifications, which may influence folding. In vivo techniques allow examination of slow and large conformational changes but miss out on the rapid formation of the secondary structure that can be seen in vitro. Resolution at the atomic level has been reserved for the test tube. Technology is being developed to cross the bridge, and insightful NMR studies on nascent chains hanging off ribosomes are a beautiful example (10). NMR also allows examination of biomolecules in intact cells, and after successful in vivo NMR studies on RNA and DNA (11), NMR on proteins in intact cells is bound to follow soon. Fluorescence techniques, such as Förster resonance energy transfer, are being used to study protein folding in bacteria and within the ribosomal tunnel, and purified-protein refolding studies have expanded to include complex cellular extracts. Techniques that allow examination of protein conformation in intact cells, such as limited proteolysis (12), antibody recognition (13), or disulfide bond formation (14–16), can and should be applied to purified proteins folding in isolation.

Evolution

The investment a cell makes for folding of proteins is enormous. Why did folding of proteins not evolve to be simpler, more fail-safe? The challenge may lie in the fact that protein evolution involves folding as well as function, two unrelated parameters in this context: A protein with improved function does not necessarily fold better. In fact, folding and function are described as opposing, competing evolutionary pressures, where functional evolution may occur at the expense of folding (17). By contrast, a protein with improved folding, in particular a protein that folds in the ER, is very likely to improve function, perhaps not on a per molecule basis but simply because more folded molecules leave the ER. Work from the Lindquist lab (18) demonstrates that protein folding not only directly but also indirectly—through the molecular chaperone Hsp90—plays a dominant role in cellular evolution. These opposing evolutionary forces, folding and function, have major consequences for new or improved protein design, as functional improvement of, for instance, an active site in an enzyme may well lead to a lower folding yield and more aggregation of the enzyme (17).

WHEN DO PROTEINS BECOME CORRECTLY FOLDED?

Proteins start to fold as soon as space allows. The ribosomal tunnel and Sec61 translocon accommodate α-helix formation (19), but conformational freedom is expanded greatly upon entry into the ER lumen. The growing nascent chain enters a sheltered environment of ER chaperones and folding enzymes

surrounding the translocon; this environment limits conformational freedom and hence slows down folding but also keeps the nascent chain away from neighboring chains from the same polysome. The chance of aggregation is highest between partially folded proteins with identical sequence as specific interactions that normally exist within the folded protein are likely to arise between folding proteins, a process called domain or strand swapping (20, 21). Next to misfolding and aggregation, incompletely folded proteins run the risk of degradation, but except for the fairly specific signal peptidase and signal peptide peptidase, proteases are absent from the ER.

Translation Rate and Folding

Cotransl(oc)ational folding rates are limited by the rate of translation. Compared to diffusion and secondary structure formation, translation is exceedingly slow, measured for individual eukaryotic proteins at 4–5 residues per second (14, 22), whereas ribosome profiling suggests orders of magnitude variation in translation rates (23). Although in vitro refolding of proteins is measured at the submillisecond timescales, folding of proteins in intact eukaryotic cells may take minutes to many hours. This comparison is not fair though as in vitro folding allows assessment of changes in secondary structure, whereas biological systems focus on the slower tertiary and quaternary structure formation. In fact, most initial secondary structure formation is faster than translation and hence happens on the nascent chain.

It is no surprise then that evidence is mounting that translation rate affects protein folding and conformation (**Figure 1**) (24). Silent substitution of a rare codon for an abundant codon or vice versa does not change the protein sequence but may affect its ability to attain the correct functional structure (25, 26). Whether codon abundance correlates with differences in

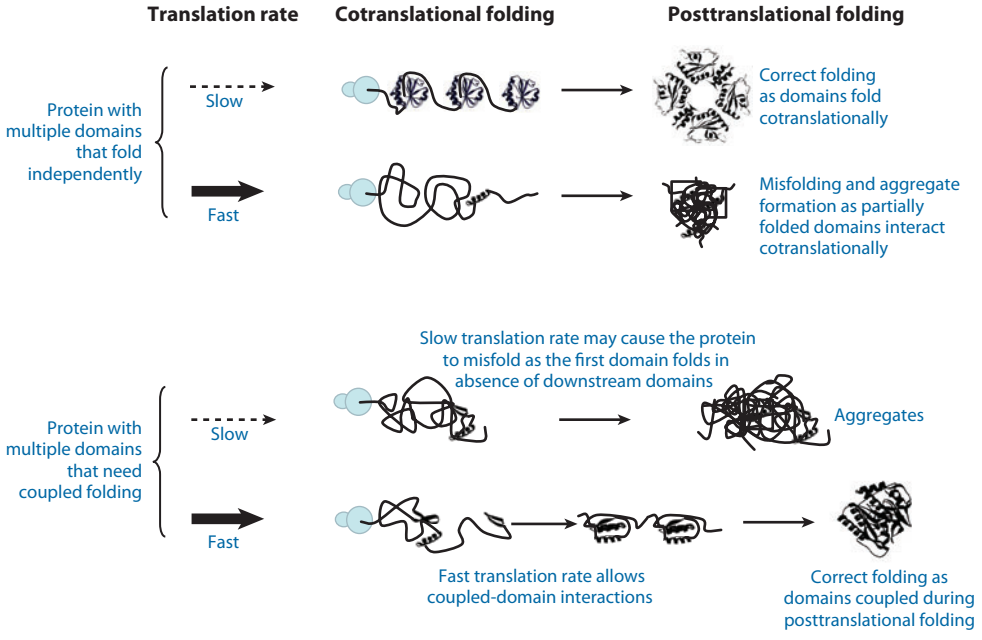

Figure 1

Effect of translation rate on folding of multidomain proteins. Proteins start to fold during their synthesis. When synthesis is slow, sequential domains have time to fold one by one. When translation is fast, a second domain may be made before the first one is folded, allowing interactions between two partially folded domains. Proteins with coupled domain folding benefit most from fast translation. Proteins with independent (uncoupled) domain folding benefit most from slow translation.

Cotranslational folding: folding of the protein while still a nascent chain on the ribosome

Uncoupled domain folding: independent folding of sequential domains in a protein

Posttranslational folding: folding of the protein after chain termination

Coupled domain folding: domains in a protein interact only during the folding process and are not associated with each other in the native structure

amino acyl tRNA concentrations in all eukaryotic cells remains to be seen, but a shortage of amino acyl tRNA does cause pausing of the ribosome on the mRNA, giving the nascent chain time to fold before downstream sequences are made (24, 25). The role of translation rate in protein folding is underscored by findings that truncated nascent chains of increasing length can reach different conformations (27) and that multidomain eukaryotic proteins often do not fold well in prokaryotic cells, where the translation rate is much higher (28). Rapid translation limits the time for cotranslational folding and allows partially folded sequential domains to interact. For independently folding domains in multidomain proteins, as in artificial multidomain proteins, a short cotranslational folding period may be detrimental for protein folding and structure (28, 29). For sequential domains (or repeats) in their natural context, however, increased interdomain interactions may well be beneficial for folding (30), implying opposite requirements for proteins with coupled or uncoupled domain folding (**Figure 1**).

Posttranslational Folding

Protein folding starts during synthesis, as a vectorial process. Co- and posttranslational folding is a continuum though, and for most proteins, the timing of chain termination may not be crucial. Influenza virus hemagglutinin (HA), for instance, does form its first two disulfide bonds during synthesis, but the subsequent set can form either during or after translation (31). HA can even postpone disulfide formation completely until after chain termination (13). Indeed, most newly synthesized proteins tested can tolerate the postponing of disulfide bond formation until after chain termination and full translocation: vesicular stomatitis virus (VSV) G protein, low-density lipoprotein (LDL) receptor, tissue plasminogen activator, immunoglobulins, thyroglobulin, procollagen, and yeast carboxypeptidase Y. The protein's final conformation, i.e., the outcome of folding, is not affected, suggesting that, for these proteins, cotranslational disulfide bond formation is not crucial. Keeping a protein reduced of course does not mean that it does not acquire conformation. Hence, folding without disulfides may be sufficient to sustain the vectorial nature of the process. By contrast, many proteins have their N and C termini close together in the three-dimensional structure, underscoring the need for posttranslational folding.

Counterintuitively, even a vectorial domain organization does not imply a requirement for cotranslational disulfide bond formation. The LDL receptor has an extended structure and a modular, sequential domain organization, like beads on a string. Still, the 40-residue N-terminal repeats only acquire their native conformation 15–30 min after chain termination, after a phase during which nonnative disulfide bonds between repeats are abundant (30). During protein refolding in the test tube, large proteins have been shown to first collapse to bury hydrophobic side chains, forming native as well as transient nonnative contacts in the protein. In vitro the denatured LDL receptor repeats refold efficiently following isolation from inclusion bodies (32), suggesting that they do have intrinsic capacity to fold on their own. In the full-length protein, however, repeat domain folding is coupled (**Figure 1**). The LDL receptor family of proteins, and other similarly organized proteins with coupled domain folding, likely benefits from rapid translation, which allows maximum interdomain interactions at the expense of nonproductive intradomain folding. This type of protein may have coevolved with the chaperone machinery. Interaction with folding factors further delays folding, thereby preventing any nonproductive interactions occurring cotranslationally and maximizing coupled posttranslational folding. Indeed, proteins with a well-characterized folding pathway in the ER show significant, long posttranslational folding phases. As is the case with protein refolding in vitro, the majority of in vivo folding involves a protein that already has its C terminus; perhaps in vitro and in vivo protein folding are not that different after all.

Special cases of protein folding in the ER include proteins with multiple membrane spans.

They need cotranslational integration of their transmembrane helices and are likely to need coordinated chaperone assistance in three compartments: the cytosol, membrane, and ER lumen. ATP-binding-cassette transporters, like CFTR, have a second ER targeting sequence in their second membrane-spanning domain (transmembrane helices 7–12), which suggests that translation of the cytosolic domains between the two membrane-spanning domains may result in, or at least allows, transient release of the ribosome from the translocon in the ER membrane. Indeed, the domains in CFTR already fold during synthesis (12). The time taken to make a CFTR polypeptide is relatively long though, ~9 min, with 1,480 amino acids and an overall translation rate of 2.7 residues per second, which is slower than the 4–5 residues per second measured for soluble proteins (14, 22). Whether the slow translation is the result of ribosome stalling caused by the second signal peptide or by pauses at many sites is unclear. Feedback regulation of the ribosome translation rate by protein folding is plausible because the electrostatic potential of the nascent chain in the ribosomal tunnel influences the translation rate (33), and rare codons often cluster downstream of domain boundaries.

The Role of the Endoplasmic Reticulum and Its Folding Factors

Although the influence of the translation rate stops at chain termination, ER-resident chaperones and folding enzymes prevent, delay, and otherwise modulate folding both during and after translation. Folding factors as well as translation rate provide time for the nascent protein to fold. Not only are interactions within the protein delayed because chaperones compete for binding, but folding factors allow a protein to backtrack on its folding path, prolonging the duration of equilibrium with a less folded state, thereby preventing trapping in a nonnative state. Similar to cotranslational folding, regulation of the kinetics of posttranslational folding is particularly important for multidomain proteins and for proteins or domains with large contact distances. N-terminal sequences need to acquire their native fold together with downstream sequences, and postponing native folding of a (sub)domain in that case is crucial for avoiding misfolding and aggregation. Manipulation of translation rates may not suffice, and the folding factors add orders of magnitude to the regulation of folding rates.

Folding starts during translation, but when does it end? Typically, newly synthesized proteins in the ER assemble into oligomers only after monomers have folded to near-native structures (34). This seems different in the cytosol, where hetero-oligomerization of two differently tagged versions of the same (normally homo-oligomeric) protein often is inefficient, which suggests assembly during translation. Cotranslational assembly may be a requirement for many cytosolic proteins as release from the ribosome lowers the effective concentration of the assembly-competent subunits by several orders of magnitude. In the ER, proteins are tethered to the membrane during translocation, and membrane proteins remain tethered; this limits their freedom of movement in the third dimension and still allows effective posttranslational assembly. Although membrane tethering may play a role in oligomer assembly, the folding process does not appear to be influenced by it: a range of proteins showed similar folding and folding assistance whether they had their transmembrane domain or a glycosylphosphatidylinositol anchor (35).

Folding After the Endoplasmic Reticulum

Is there folding beyond the ER? The ER is supposed to exert quality control on its newly synthesized proteins and to allow exit of only properly folded and assembled proteins. The first requirement for exit is the release from resident ER chaperones and folding enzymes. Folding intermediates, misfolded aggregated proteins, assembly-competent oligomer subunits, and orphan subunits of hetero-oligomers as a rule remain in the ER, but examples do exist of proteins changing conformation in

Client proteins: proteins destined for secretion that interact with ER folding factors. For enzymes, they are called substrate proteins

PPIases: peptidyl prolyl *cis/trans* isomerases

the Golgi complex or beyond. For instance, large complex proteins that assemble into tightly packed structures in secretory granules, such as secretogranins (36), mucins, and the von Willebrand factor (37), do undergo conformational changes after exit from the ER. Even though intrachain disulfide bond formation is predominantly an ER-catalyzed process, interchain disulfides, in principle, can form after exit (36), but for most proteins, this question simply has not been addressed.

Strong evidence for the folding factors acting in the ER and not beyond is the frequent observation that newly synthesized proteins are malleable and sensitive to unfolding while still in the ER but acquire resistance to unfolding treatments upon exit, irrespective of whether the protein is still inside the cell, has been secreted, or is even purified. Examples include the resistance to reduction by dithiothreitol (38), oxidation by diamide, calcium depletion (39), aggregation by ATP depletion (40), degradation by proteases, and nonpermissive temperature for temperature-sensitive (ts) mutants (41). The chaperones are the likely cause for this sensitivity. As long as chaperones and folding enzymes act on a protein, that protein remains sensitive to conformational changes. Once released from these helpers, exit may be rapid, and the protein may be compact and relatively resistant to insult.

While ER-resident proteins reside in the ER, they may cycle through the earlier stacks of the Golgi [or rather the ER-to-Golgi intermediate compartment (ERGIC) or *cis*-Golgi network] via the KDEL receptor, either unbound or with client proteins attached. In the ERGIC, a second level of quality control exists. The well-known ts mutant of VSV G protein, *ts045*, can reach the ERGIC at the nonpermissive temperature, but upon further insult (reduction by dithiothreitol) immediately stops cycling and is retained in the ER (42). Cargo is not only sorted to Golgi or back to the ER by this compartment but a third target is an endosomal compartment that allows secretion of proteins while bypassing the Golgi altogether (43, 44). Finally, endosomal compartments, *trans*-Golgi network, as well as the plasma membrane sort proteins for different destinations based on the protein's age or stability. For instance, mutant CFTR in the plasma membrane is subject to quality control and degradation (45), suggesting recognition but perhaps also regulation of protein conformation well beyond the ER. Perhaps the question should not be "When does folding end?" but rather "Does folding ever end?" And the answer is no. Proteins keep changing their conformation during receptor-ligand interactions, during enzymatic reactions, and also during activity cycles, phosphorylation cycles, and assembly-disassembly cycles. Molecular chaperones, such as Hsp70s and Hsp90s, are not done with proteins after they have reached their native conformation but may assist conformational changes throughout a protein's lifetime.

WHAT CELLULAR PROTEINS ARE INVOLVED IN PROTEIN FOLDING?

The search for folding enzymes or chaperone proteins in the ER has provided us with a comprehensive list of proteins, which either have been shown to influence folding reactions directly or are assumed to play some role in the folding process owing to their homology with other folding factors. A list of these proteins is presented in **Table 1**; the categories of folding factors are based upon whether they catalyze specific steps or are able to bind to intermediates in the folding pathway. For convenience, the general folding factors are divided into heat shock proteins acting as chaperones or cochaperones, peptidyl prolyl *cis/trans* isomerases (PPIases), oxidoreductases, and glycan-binding proteins. In addition to the general folding factors that have a number of client proteins, there are also factors that act on a limited subset of client proteins. These personal or private chaperones must have evolved to solve a problem in the maturation of a particular protein or protein domain and are included along with an indication of their client protein.

Multifunctional Proteins and Redundancy

There are a couple of striking features that can be deduced from the list of folding factors. It is clear that some factors are able to perform multiple functions, and in addition, there seems to be a great degree of functional redundancy with several classes of proteins having overlapping functions. For example, the protein BiP is involved in gating the translocation apparatus (46), regulating the unfolded protein response (UPR) (47), targeting proteins for degradation (48), as well as recognizing hydrophobic domains within folding protein domains thereby stabilizing folding intermediates (49). Likewise, the protein PDI not only catalyzes disulfide exchange reactions but also acts transiently as a polypeptide-binding protein to stabilize intermediates in the assembly of multisubunit complexes (50), forms stable interactions with polypeptides prone to aggregation thereby becoming a noncatalytic partner (51), and facilitates the targeting of proteins for degradation (52). Functional redundancy becomes apparent when you consider the several PPIase and Protein Disulfide Isomerase (PDI) family members. The ER-localized PPIases are currently poorly characterized, but with so many enzymes able to catalyze the same reaction, the likelihood of functional overlap is high. Indeed, the substrate specificity of the PDI family of enzymes demonstrates some limited specificity (53, 54) but also indicates that loss of one enzyme can be compensated by other family members (55). Our understanding of the role of each folding factor in the maturation of individual client proteins within the ER is complicated by this functional redundancy. In addition, the requirement of several factors to act in concert during the maturation process means that it is difficult to deconvolute the role each factor plays. So, although we can demonstrate that under certain circumstances folding factors can carry out a particular role, concluding that they do carry out the same role in the cell in a particular tissue is wrong.

A Folding Pathway

Despite these limitations, the detailed folding pathway of some proteins has been delineated along with the specific factors required for maturation. Perhaps the best way to describe protein folding in the ER is to focus on a well-characterized example. This does run the risk of oversimplifying what is clearly a very complex process with lessons learned from one maturation pathway difficult to apply to other client proteins. It is also clear that the folding of homologous protein domains can require specific folding factors, a point that is clearly demonstrated by the folding of immunoglobulin G (IgG) (56). The IgG example also highlights the concept that folding factors do not work in isolation; they are likely parts of complexes, which work in concert to optimize folding reactions.

IgG is assembled from two light and two heavy chains, which consist of two and four, respectively, structurally similar IgG domains (57). The complete tetramer needs to fold and assemble prior to secretion from cells. The folding of the heavy chain is critical to allow secretion to occur as unassembled heavy chains interact with BiP, resulting in their retention within the ER (58). It is known that the second Ig domain within the heavy chain, called the C_H1 domain, mediates this interaction (59). In contrast to all other Ig domains so far studied, the C_H1 domain does not fold when synthesized in isolation (60). Remarkably, for this domain to fold correctly, it must first form an internal disulfide and then interact with the C_L domain, which is its cognate partner in the antibody molecule. In addition, studies on the folding of the domain in vitro reveal the requirement of a PPIase to accelerate proline isomerization and folding of the C_H1 domain once the C_H1/C_L heterodimer has formed (60). Finally, a second interchain disulfide forms between the C_H1 and the C_L domains. These in vitro studies revealed that BiP can bind to the unfolded C_H1 domain with or without its disulfide but does not bind to the C_H1/C_L heterodimer. The requirements for disulfide formation, C_L assembly, proline

UPR: unfolded protein response

PDI: protein disulfide isomerase

IgG: immunoglobulin

Table 1 Folding factors resident in the endoplasmic reticulum (ER) of mammalian cells

Family	Folding factor	Function	Client proteins or their conserved properties
		Chaperones	
Hsp90	Grp94	An essential protein for secretion of specific clients and ERAD of some proteins	IGF, IGF I, IGF II (119) ERAD α1-antitrypsin (120)
Hsp70	BiP	Translocation into the ER Unfolded protein response Protein folding ERAD Holds Ig HC until displacement by light chain for Ig assembly (121)	Broad specificity; recognizes heptapeptide sequences with a high hydrophobic content (49) Narrow specificity; immunoglobulin domains (121)
		Cochaperones	
Hsp110	GRP170	Nucleotide exchange factor for BiP (65, 122, 123)	BiP
	BAP/SIL1	Nucleotide exchange factor for BiP (122, 123)	BiP
Hsp40	ERDJ1	BiP cochaperone; protein biosynthesis (180)	BiP and α1-antichymotrypsin, inter-α-trypsin inhibitor HC (124, 125), among others
	ERDJ2/SEC63	BiP cochaperone; translocation; associated with polycystic liver disease (126)	BiP and translocating proteins
	ERDJ3	BiP cochaperone (127, 181); protein folding and ERAD	BiP and translocating proteins
	ERDJ4	BiP cochaperone; ERAD	BiP and surfactant protein C (128), among others
	ERDJ5	BiP cochaperone; potential disulfide reductase involved in ERAD (128, 129)	BiP and misfolded proteins
	ERDJ6	BiP cochaperone; defends cells against ER stress	BiP and misfolded proteins (130)
		Peptidyl prolyl *cis/trans* isomerases	
Cyp	Cyclophilin B	PPIase	Procollagen (131)
FKBP	FKBP2	Potential PPIase	Unknown
	FKBP7	Associates with BiP, PPIase (132)	Unknown
	FKBP9	PPIase (133)	Unknown
	FKBP10	PPIase	Procollagen (134)
	FKBP11	Pancreas-specific PPIase (135)	Unknown
	FKBP14	Potential PPIase (by homology)	Unknown
		Oxidoreductases	
PDI	PDI	Formation, isomerization, and reduction of disulfides, potential chaperone (50), ERAD (52)	Broad specificity (136)
	ERp57	Calnexin/calreticulin-associated oxidoreductase (99)	Glycoproteins. Isomerization of nonnative disulfides in cysteine-rich unstructured domains (53)
	ERp72	Potential oxidoreductase (137)	Thyroglobulin (138), ERp57 substrates when calnexin/calreticulin cycle is blocked (55)
	P5	Potential oxidoreductase (139); associates with BiP and forms mixed disulfides with BiP client proteins (54)	BiP client proteins (54)
	PDIp	Pancreas-specific PDI	
	PDIR	Potential oxidoreductase (140)	Unknown
	ERp46	Protection against ER stress (141, 142)	Insulin, adrenomedullin, endothelin-1 and CD105 (141, 142)

(*Continued*)

Table 1 (*Continued*)

Family	Folding factor	Function	Client proteins or their conserved properties
	ERp18	Potential oxidase (143, 144)	Unknown
	ERp29	Molecular chaperone preventing premature oligomerization (145) and stimulating secretion	Connexin43 (145), thyroglobulin (182), Pipe (183)
	ERp27	Peptide binding (146)	Unknown
	ERp44	Thiol-dependent retention in the ER (147–151), inositol 1,4,5-triphosphate receptor regulation (152)	IgM, Ero1α, SUMF1, formylglycine-generating enzyme, adiponectin (147–151)
	PDILT	Testis-specific PDI forms a complex with calnexin homolog, calmegin (153, 154)	Unknown
	TMX1	Protection from stress (155); potential function in quality control (156)	MHC class I HC (156)
	TMX2	Oxidoreductase (by homology)	Unknown
	TMX3	Potential thiol oxidase (157)	Unknown
	TMX4	Oxidoreductase (by homology)	Unknown
	AGR2	Potential oxidoreductase	Mucins (158)
	pERp1	Oxidative protein folding (159, 160)	Immunoglobulins (159, 160)
Sulfydryl oxidase	Ero1α	Oxidase, regulation of redox conditions in the ER (161–163)	PDI family members (54)
	Ero1β	Oxidase, induced by the UPR, high expression in the pancreas (164, 165)	Presumed to be PDI family members
PDI-Erv	QSOX1	Thiol oxidase (166)	Most protein thiols (167)
	QSOX2	Thiol oxidase (by homology) (168)	Unknown
Peroxiredoxin	PrxIV	H_2O_2 metabolism and thiol oxidase (169, 170)	PDI family members
		Glycan-binding proteins	
Calnexin family	Calnexin	Binds glycoproteins via monoglucosylated N-linked oligosaccharides leading to ER retention (100)	Glycoproteins at early stages of synthesis (31)
	Calreticulin	As calnexin, soluble rather than membrane associated (100)	Glycoproteins (66)
	Calmegin	Testis-specific homolog of calreticulin (171)	Testis glycoproteins (171)
UGGT		Reglucosylation of incompletely folded glycoproteins	Glycans attached to nonnative protein domains (172)
Ost6p and Ost3p		Oxidoreductase activity affecting glycosylation efficiency (173)	Glycoproteins
		Client-specific folding factors	
	P4H	Chaperone for unhydroxylated triple helical domains (72)	Procollagen
	Hsp47	Prevents premature association of procollagen monomers in the ER	Procollagen
	RAP	Facilitates folding of LDL receptor family members (174)	LRP1, megalin and VLDLR
	Mesd	Aids secretion of LDL receptor family members	LRP 5/6 (175)
	MTP	Assists assembly and secretion of apoB-containing lipoproteins (176)	Apolipoprotein B
	Tapasin	Ensures loading of high-affinity peptides to MHC class I (177)	MHC class I
	Invariant chain	Prevents aggregation of α and β chains of MHC class II (178)	MHC class II
	Cosmc	Prevents aggregation and degradation (179)	T-synthetase

ERAD, endoplasmic reticulum–associated degradation; HC, heavy chain; LDL, low-density lipoprotein; MHC, major histocompatibility complex; PDI, protein disulfide isomerase; UPR, unfolded protein response.

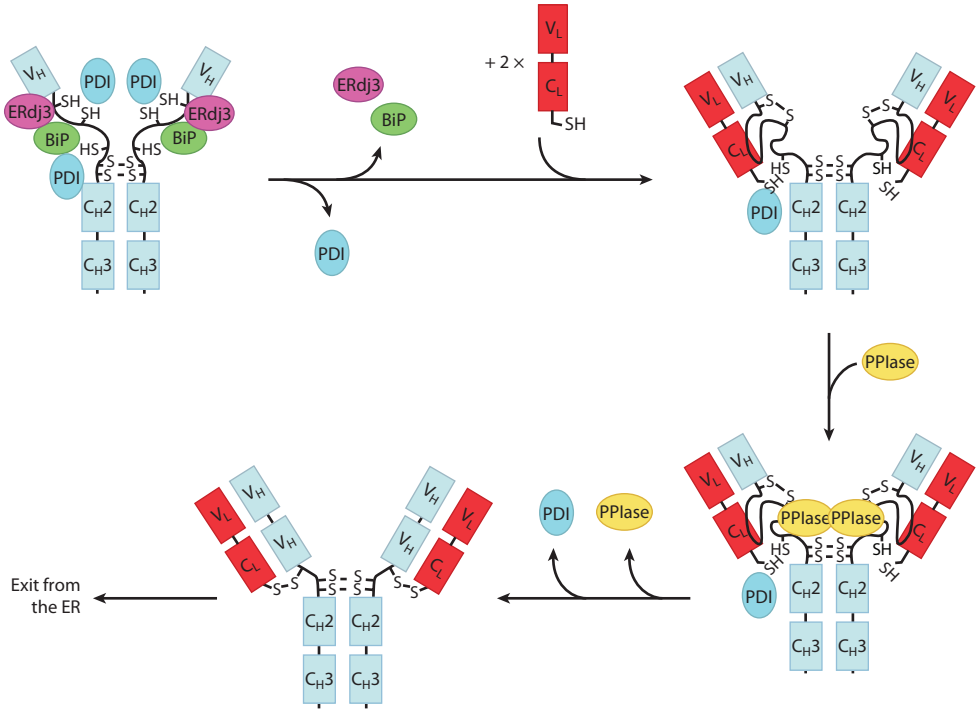

Figure 2

Folding of immunoglobulin G (IgG) C_H1 domain requires several folding factors that act sequentially. The final stage of folding and assembly of the IgG heavy and light chains is initiated by the association of two light chains with a heavy-chain dimer in which the C_H1 domain is unfolded and stabilized by interaction with the folding factors BiP, ERdj3, and PDI. These folding factors dissociate upon light-chain association. C_H1 folding then is catalyzed by an endoplasmic reticulum (ER) peptidyl prolyl *cis/trans* isomerase (PPIase) (60). PDI catalyzes both intrachain and interchain disulfide formation. In vivo the interactions may be more parallel than the cartoon portrays. Once fully assembled, the IgG molecule will exit the ER and will be secreted from the cell. BiP, immunoglobulin-heavy chain binding protein; C_H1, C_H2, and C_H3, constant domains of immunoglobulin heavy chain; C_L, constant domain of immunoglobulin light chain; ERdj3, endoplasmic reticulum J-domain protein 3; HS and SH, free thiol groups; PDI, protein disulfide isomerase; S-S, disulfide bond; V_H, variable domain of immunoglobulin heavy chain; V_L, variable domain of immunoglobulin light chain.

isomerization, and interaction with BiP are all necessary for the correct folding and secretion of the C_H1 domain in cells (60). Hence, for the folding of this particular Ig domain, there is a requirement for at least four folding factors: an oxidoreductase, a PPIase, BiP, and its cochaperone ERdj3 (**Figure 2**).

Regulation of Folding Factor Activity

The folding of IgG also illustrates the possibility that complexes of folding factors may form to facilitate the folding of specific client proteins. Recent evidence suggests that P5 may be the PDI family oxidoreductase responsible for disulfide formation in IgG (54). P5 also forms a noncovalent interaction with BiP (54, 61), suggesting that the specificity of P5 for its clients could be because of its interaction with BiP. A similar situation arises for a second PDI family member ERp57 (62). Here, substrate specificity is linked to a noncovalent interaction with the ER proteins calnexin and calreticulin. These ER lectins bind to newly

synthesized glycoproteins and in doing so target ERp57 to glycoproteins. It may well be that PPIases as well as PDI family members are targeted to substrates in this way. For both BiP and calnexin/calreticulin, the initial recognition of the client protein is important, not only for chaperone function, but also for the targeting of specific enzymes involved in accelerating protein folding.

For chaperone proteins to function effectively, they must be able to regulate their binding to client proteins. For the Hsp70 proteins, such as BiP, this regulation involves a cycle of binding and release brought about by conformational changes in the protein following ATP hydrolysis. In general terms, Hsp70s, such as BiP, function by first binding to hydrophobic regions of client proteins (49) in an ATP-bound state through low-affinity interactions, which become more stable upon ATP hydrolysis (63). The J domain of an Hsp40 cochaperone binds to BiP and accelerates ATP hydrolysis (64), thus stabilizing client binding. A nucleotide exchange factor, such as GRP170 (65), then induces the release of ADP from BiP causing the complex with client protein to dissociate, thereby completing the cycle. By merely binding to hydrophobic regions of polypeptides, BiP allows proteins to fold more efficiently by protecting these regions from nonproductive associations. Once the hydrophobic regions are internalized into the native protein structure, then BiP no longer binds, allowing correctly folded proteins to exit the cycle.

The binding of the ER lectins calnexin and calreticulin is regulated by trimming of the oligosaccharide side chain: Binding and release are determined by monoglucosylation of the sugar chain (66). Exit from the cycle occurs once the side chains are no longer monoglucosylated, a process that is regulated by glucosidases and the enzyme UDP-glucose glycoprotein glucosyl transferase (UGGT) (67). UGGT has the ability to specifically reglucosylate incompletely folded clients, ensuring that escape from the calnexin/calreticulin cycle only happens following correct folding (68). By restricting specificity to a particular oligosaccharide structure, calnexin and calreticulin act to both retain unfolded proteins in the ER and to target ERp57 to polypeptides during their folding. This may be particularly important for glycoproteins with cysteine-rich unstructured domains in need of an isomerase to unscramble any nonnative disulfides (53).

Other examples of mechanisms for regulating binding of folding factors to client proteins come from the client-specific factors. Hsp47 is thought to bind to procollagen chains to prevent lateral association prior to transport to the Golgi (69). Binding of Hsp47 to procollagen is regulated by pH, suggesting that the more acidic conditions of the Golgi allow Hsp47 to dissociate and to be transported back to the ER (70). Proyl4-hydroxylase modifies proline residues in the procollagen triple helical domain to hydroxyproline and in doing so allows the triple helical domain to be stable at 37°C (71). In the absence of cofactors such as ascorbate, iron, or oxygen, the enzyme binds tightly to the unhydroxylated chains, thereby limiting misalignment of the individual chains that ultimately form the triple helix (72). Once the enzyme has catalyzed proline hydroxylation, it has a much lower affinity for the hydroxylated chains, which allows the triple helix to form. Finally, tapasin binds to major histocompatibility complex (MHC) class I heavy chain following its assembly with β2-microglobulin, allowing the formation of a peptide-loading complex with the peptide transporter, calreticulin, and ERp57 (73). Binding of tapasin to heavy chain is reversed upon binding of the optimal peptide to the heavy chain by a process that likely involves a conformational change in the class I molecule (74). In each of these examples, the common characteristic of the folding factor is that it can recognize the client protein only when it is unfolded or incompletely assembled. By simply binding to its client protein, the factor can both aid the efficiency of protein folding and, as we discuss below, restrict trafficking out of the ER.

Calnexin/calreticulin cycle: the cycle of glycoprotein binding and release via their glycan from a complex containing calnexin/calreticulin and ERp57

Nonnative disulfide: a disulfide bond not present in the native structure that forms in a protein either during folding or when misfolding

Endoplasmic reticulum–associated degradation (ERAD): a process that targets misfolded proteins for degradation in the cytosol by the proteasome following retrotranslocation from the ER

ER folding factors: resident ER proteins that assist in protein folding by binding or by catalyzing covalent reactions during the folding process

COMPETING PROCESSES DURING PROTEIN FOLDING

The folding of a protein in the ER is intimately linked to both its trafficking from the ER and its degradation via endoplasmic reticulum–associated degradation (ERAD). Following entry into the ER, proteins collapse to form an ensemble of folding intermediates; some of these fold productively, and others either aggregate or are targeted for degradation (see **Figure 3**). The proteins that fold correctly are ready for anterograde transport, whereas the intermediates that do not fold correctly are prevented from secretion by their aggregation or interaction with ER folding factors, or by a combination of the two. The balance between folding, aggregation, and degradation determines the efficiency of secretion. Proteins that are compromised in folding in some way, for example, by destabilizing point mutations, are retained in the ER and inefficiently secreted. For a protein to be secreted efficiently, competition between folding, aggregation, and degradation must be such that folding occurs most rapidly. The interaction of folding intermediates with ER folding factors sequesters the protein away from the degradation pathway, possibly in complexes, thereby allowing it more time to fold correctly. Some good examples of noncovalent and covalent complexes between aggregated folding proteins and folding factors exist, for example, during the folding of thyroglobulin (75) or of the VSV G protein (76). The ability to form these higher-order structures may well provide the folding proteins a mechanism to avoid the degradation pathway, which, owing to the requirement to transport the polypeptide to the cytosol for proteasomal attention, requires the substrate for degradation to be monomeric and possibly unfolded.

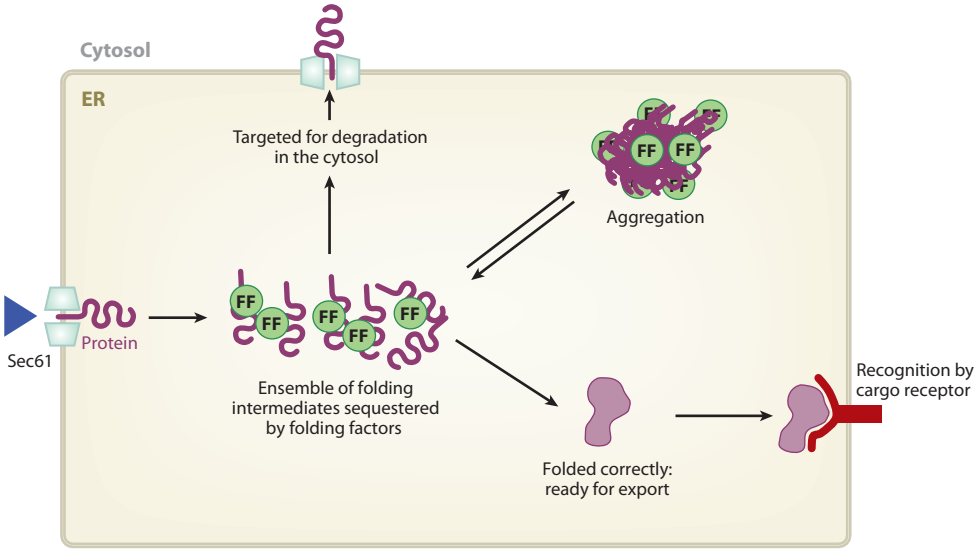

Figure 3

Competition between folding, aggregation, and degradation. Proteins enter the secretory pathway via Sec61 (*blue arrowhead*) and begin to fold with the help of endoplasmic reticulum (ER) folding factors (FFs). Proteins that do not fold correctly self-associate to form aggregates and in the process sequester folding factors. Nonnative folding intermediates may be targeted for degradation if their folding to the native structure is delayed or hampered. Degradation takes place in the cytosol, so targeted polypeptides must be translocated out of the ER. Aggregated proteins do not enter this pathway, although they may become degraded following autophagy. Correctly folded proteins exit the ER, usually following recognition by cargo receptors, which concentrate at ER exit sites and form COPII-coated transport vesicles for delivery of the cargo to the ER-to-Golgi intermediate compartment and the Golgi apparatus.

However, folding intermediates that fail to fold or that become sequestered with folding factors or in aggregates are ultimately targeted for degradation.

Such a balance between folding, aggregation, degradation, and secretion has been termed proteostasis and is aptly demonstrated in the case of specific proteins, such as bovine pancreatic trypsin inhibitor and transthyretin, whose secretion efficiency correlates with thermodynamic and kinetic stability (77–79). In addition, the competition between folding and secretion versus degradation can be tipped in favor of folding either by stabilizing the protein structure using small molecules (or cofactors) or by increasing the concentrations of folding factors in the ER (80). For example, the addition of thyroxine, which binds and stabilizes transthyretin, increases secretion (77), and induction of the UPR, which increases ER folding factors, ameliorates the poor secretion of glucocerebrosidase variants (80). Interestingly, tissues that have high intracellular concentrations of thyroxine secrete mutant transthyretin more efficiently than tissues with low intracellular concentrations, leading to tissue-specific effects of the mutant protein (77). However, blocking degradation does not necessarily lead to more efficient secretion, demonstrating that degradation usually is downstream from a block in secretion of misfolded proteins (79). Nonetheless, the ability to manipulate proteostasis has the potential to overcome some of the protein folding diseases caused by the retention of potentially functional protein in the ER.

It has also been noted that the rate of aggregation of proteins inversely correlates with their levels of expression, indicating that proteins that are particularly prone to aggregation are expressed at relatively low levels (81). The consequence of this observation is that cells can only barely cope with the level of expression of certain proteins; if this level were to rise, these proteins would have a tendency to aggregate, resulting in the formation of toxic inclusions. The balance of competing processes that take place during protein folding in the ER therefore is also influenced by the levels of individual protein expression.

Mechanism of Endoplasmic Reticulum Retention

The sequestering of proteins away from the degradation pathway by interaction with ER folding factors can be considered the main mechanism for the retention of nonnative proteins in the ER. The mere binding of folding intermediates to the ER-resident proteins, such as calnexin, calreticulin, BiP, or PDI, will both protect the intermediate from ERAD and prevent secretion. This concept, although appealing, does not take into account any potential regulation of trafficking at the level of cargo recognition. Once folded proteins are released from the ER folding factors, they may also be subject to selection for transport. In most instances, proteins are recognized by cargo receptors in the ER to allow subsequent concentration of the cargo at ER exit sites, followed by the formation of COPII-coated vesicles and transport to the Golgi apparatus (82–87). ER-resident proteins may be excluded from such vesicles, thereby preventing their transport to the Golgi apparatus. There is a slow release of ER-resident proteins into COPII vesicles, requiring a mechanism for their retrieval from the Golgi apparatus (88). In addition, proteins expressed at high levels that are still able to fold correctly may also be transported by a receptor-independent mechanism (89). That said, for most proteins, a combination of release from ER-resident proteins and cargo receptor recognition is required for efficient secretion.

The combined ability of ER folding factors to recognize folding intermediates and the recognition of correctly folded proteins by cargo receptors indicates that the ER exerts a system of quality control over proteins destined for secretion (90). This concept does not necessarily mean that the ER can in some way sense whether a protein has attained a functional state. There are many examples where proteins with nonfunctional conformations are secreted (91), whereas other proteins have

COPII-coated vesicles: transport vesicles that bud off from the ER and carry cargo proteins via an intermediate compartment to the Golgi apparatus

attained a functional state and are retained. Membrane proteins may be retained in the ER as well, even when the major part of the protein folds in the cytosol rather than the ER. In these cases, it is probably more appropriate to suggest that the quality control system in the ER can in some way discriminate between molecules with differing thermodynamic stabilities. Those that attain this stability without attaining functionality owing to a mutation in, for instance, a substrate-binding site may still be secreted. Likewise, some proteins could be functional but for some reason have not attained structural stability and are therefore retained in the ER.

THE CONSEQUENCE OF POLYPEPTIDE CHAIN MODIFICATION FOR PROTEIN FOLDING

Several modifications occur to a polypeptide chain both during and after its synthesis and translocation into the ER. Many of these have consequences on the folding of the polypeptide chain, and in addition, the folding of the chain can influence the particular modification. Here, we focus on a few specific examples where there is clear evidence of an effect that comes about either as a consequence of a change to the physical properties of the polypeptide or by targeting the chain to a specific set of folding factors.

Signal Peptide Cleavage

In most instances, the cleavage of the N-terminal signal sequence occurs very rapidly after the appearance of the protein in the ER lumen. However, there are a few instances where the cleavage takes place several minutes after the polypeptide has been fully synthesized and translocated into the ER. In these instances, the presence of the signal sequence is likely to influence the kinetics of protein folding and association with ER chaperone complexes (92). The tethering of the N terminus to the membrane via the signal sequence limits the freedom of movement of the N terminus, with obvious consequences for folding of the polypeptide. The few known examples concern long signal sequences attached to viral proteins, and for HIV Envelope glycoprotein, the cleavage of the signal sequence actually requires folding of the polypeptide chain as preventing disulfide formation leads to a lack of signal sequence removal (16). Another feature of signal peptides is their differences in quality, meaning that they can be more or less efficient in targeting a protein to the ER membrane. Targeting efficiency directly relates to the expression level of a protein and may lead to differences in topology and modifications. A perhaps extreme example of this is the prion protein PrP (93). It may be less unique than perceived, however, because the roles of signal peptides and their timing of cleavage simply have not been studied for the vast majority of proteins that enter the secretory pathway. The importance of timing, location of signal peptide cleavage, and of signal peptide quality is further underscored by the identification of mutations and polymorphisms in proteins' signal peptides in disease (94, 95).

N-Linked Glycosylation

The addition of a large hydrophilic sugar moiety to the polypeptide chain has obvious consequences for protein folding. Removal of potential glycosylation sites (sequons) by mutagenesis usually, but not always, results in misfolding of the polypeptide and intracellular retention. This effect is likely to be caused by the glycan interacting with the polypeptide to prevent the exposure of hydrophobic patches on the surface of glycoproteins, thereby preventing misfolding and aggregation. Indeed, a recent glycoproteomic study has verified that most of the occupied sequons are at the surface of proteins (96), which infers that potential sequons buried in the interior of the protein would not be utilized. The oligosaccharide side chain on the protein surface hence influences thermodynamic stability of the native structure and as a consequence the protein's ability to fold correctly and be secreted. On top of this, glycans may directly influence conformation of the peptide they are attached to, an additional local

effect. The reverse applies as well: The folding of the polypeptide chain and early formation of disulfide bonds influence occupancy of potential glycosylation sites. If folding is inhibited or prevented in some way, sequon occupancy is generally increased (15, 97). Hence, there is a temporal relationship between the rate of folding and glycosylation.

In addition to its role in stabilizing the native protein structure, the presence of an oligosaccharide side chain can also target the protein to a distinct set of folding factors. As mentioned above, the presence of a monoglucosylated side chain targets the protein to the calnexin/calreticulin cycle, which both retains the unfolded protein in the ER and brings the chain into close proximity to a complex of chaperones (98), including the ER oxidoreductase ERp57 (99). In this way, the influence of the glycan extends beyond simply stabilization of the native protein structure; rather, it facilitates the folding process itself, albeit indirectly. As well as targeting proteins to complexes involved in protein folding, the oligosaccharide side chain can, after further modification, target the polypeptide to different complexes involved in ERAD (100). The timing of trimming of the side chain allows a sequential interaction first with folding factors and then with ERAD components. This allows the polypeptide a chance to fold initially but if this is unsuccessful leads to targeting of the protein for degradation.

Disulfide Bond Formation

The introduction of disulfides into polypeptides is thought to stabilize the final native structure, but for most proteins, this particular modification was shown to affect folding and secretion. Some native proteins, such as the bovine pancreatic trypsin inhibitor, indeed can be unfolded by reduction, and for these proteins, the disulfide bonds have a stabilizing function. Because disulfide bonds in most proteins cannot be reduced anymore after the protein has folded, the role of disulfides in a protein's stability rarely can be tested. Biophysics and theory teach us that, during folding, disulfide bonds indirectly stabilize the native structure by directly destabilizing the nonnative structure. Exceptions exist where the formation of a disulfide occurs as part of the regulation of the protein function, such as cell-surface receptors that switch between active and inactive forms (101, 102). The formation of disulfides occurs primarily by disulfide exchange with a member of the PDI family of oxidoreductases (103) with the modification requiring folding of the polypeptide chain. Even though disulfides may form as the polypeptide is translocated across the ER membrane, the covalent link must be between cysteines that become juxtaposed following the collapse of the chain. In some instances, this may result in the formation of nonnative disulfides, which need to be broken, and correct ones need to be formed if the protein is to fold correctly. Such a requirement for disulfide shuffling is particularly true for protein domains that are cysteine rich yet have little secondary structure (53). Nonnative disulfides could stabilize productive folding intermediates and are therefore crucial for the efficient folding of some proteins. For example, a disulfide that forms between a cysteine residue within the prosequence of bovine pancreatic trypsin inhibitor greatly facilitates folding of this protein (104). This disulfide is not present in the final native structure. In addition, the folding of the LDL receptor requires the formation of several nonnative disulfides (30). Hence, disulfide formation is one modification that is intimately linked to the folding of the polypeptide chain.

Proline Hydroxylation

Perhaps the clearest example of a polypeptide chain modification that stabilizes the final native structure is the hydroxylation of proline residues in collagenous domains. Without this modification, the triple helical domains cannot form as the melting temperature of an unhydroxylated helix is about 25°C (105). Following hydroxylation, this rises to just above body temperature, allowing the helix to form but also allowing localized unfolding, which may be

required for collagen remodeling in the extracellular matrix. Folding of the polypeptide into a helix prevents hydroxylation as the enzyme catalyzing the reaction will only bind to nonhelical chains (106). Hence, the process is self-regulatory: Once sufficient proline residues are hydroxylated to allow a stable helix to form, the enzyme can no longer catalyze further modifications, ensuring that the melting temperature of the helix is just above 37°C.

CONTROLLING THE ENVIRONMENT FOR CELLULAR PROTEIN FOLDING

The ER is a membrane-enclosed compartment that has a distinctly different milieu than cytosol. The membrane forms a barrier for both large and small molecules, except for strongly hydrophobic ones, which may diffuse through the membrane. Whether the ER is leak free is still under debate (107), but the vast majority of molecules only enter or leave the ER via regulated channels and transporters or via vesicular transport from and to other compartments.

Energy

Energy is a first requirement for many cellular processes, and the ER is no exception. ATP is used for phosphorylation and by chaperones of the heat shock families. Hsp70 BiP's function is regulated by nucleotide exchange factors and nuclease-activating factors that modulate its ATPase cycle (see above) (108, 109). However, such cochaperones have not been found yet for Grp94, in stark contrast to cytosolic Hsp90, which has at least a dozen. In addition to the molecular chaperones, solute transporters are other abundant ATP consumers; they keep the (ionic) ER milieu constant if necessary against a concentration gradient.

As ATP levels usually are measured biochemically, the ER ATP concentration has not been measured yet but is assumed to be similar to that in the cytosol. An ATP transporter was functionally identified (110), and a decade later, a candidate protein was found (111). In both studies, it was shown to be an ATP/ADP antiporter and to be distinct from the mitochondrial ATP transporter. The first candidate gene was found in plants; ANT1 encodes an ER-resident ATP-ADP antiporter that is required for maintaining expression levels of ER-resident chaperones but is not essential (112). In the large families of transporter proteins, the search for a mammalian ortholog and alternative transporters continues.

Although the ER is a single continuous network of membranes, it does consist of functional subcompartments. However, no further membrane barriers exist between the different regions (including nuclear envelope). Well-known subcompartments include the smooth ER, which lacks bound ribosomes, is filled with detoxification enzymes, and is prominent in the liver. Well-known also are the exit sites from which COPII-coated vesicles bud, the "degradation region" enriched in EDEM and other proteins involved in ERAD. More are likely to exist, with subregions of the ER in which misfolded aggregates accumulate, where different sets of chaperones and folding enzymes are enriched and perhaps even with a different pH, ATP concentration, and net redox milieu. That this is likely is illustrated by the different Ca^{2+} levels in regions of the ER, already identified in the early 1990s by the Meldolesi lab (113).

Calcium

Together with mitochondria, the ER is the major calcium storage compartment of the eukaryotic cell. Its resident proteins, in particular the molecular chaperones and folding enzymes, have both high- and low-affinity calcium-binding sites. Their isoelectric points are acidic because of the many glutamate and aspartate residues in their sequence, and in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, they usually run larger than their calculated mass predicts owing to their incomplete SDS binding. Although all known folding factors bind calcium, calnexin, calreticulin, BiP, Grp94, and PDI are considered most important for calcium storage

because of their sheer abundance and number of Ca^{2+}-binding sites.

Entry and exit of calcium from the ER are tightly regulated and well characterized. Even though entry is ensured by constant pumping of the thapsigargin-inhibited Ca^{2+}-ATPase (homologous to the sarcoplasmic reticulum calcium ATPase in the sarcoplasmic reticulum in muscle) (113), exit is mediated by controlled release through the ryanodin receptor and (as more recently found) also in pannexin channels (114). Tight control and protein mediation of entry as well as exit of this small cation argue for a leak-free ER under physiological circumstances.

That high calcium concentration in the ER is important for folding is clear, but the required levels and mechanism of its activity are under debate. Proteins that incorporate calcium in their structure usually require the cation to acquire a native folded structure. Whether folding factors like Grp94 and calreticulin require calcium for proper functioning may well depend on the method of calcium depletion. Calcium depletion by chelation releases all calcium from proteins, from low- as well as the high-affinity sites, which is likely to affect their function. Depletion in the absence of chelation, by contrast, allows calnexin, for example, to still bind its clients (115). This is consistent with physiology as during intracellular calcium waves only the low-affinity sites are emptied, and the high-affinity calcium stays bound, which may be dispensable for function.

Size and Protein Content of the Endoplasmic Reticulum

Although balanced low-molecular-weight conditions (including ATP and Ca^{2+}) are a condition *sine qua non* for proper folding, the proteinaceous conditions are needed as well. The so-called higher eukaryotes derive their complexity not from a larger number of protein players but from an abundance of interactions, essentially multiplying the diversity of "chaperone machineries" that can act on a folding protein. As mentioned above, the folding factors frequently combine to form complexes, which work in combination to fulfill folding functions. Identification of such complexes usually is done by biochemistry, which by definition implies disruption of the cell. So far, identical folding factor complexes have not been found in different studies, and diffusion rates of GFP-tagged resident folding factors appear high (116), suggesting that these complexes may be transient in nature and may depend on the client. The subregions of the ER hence may be highly dynamic as well, and all the more difficult to trap and define.

An additional level of regulation concerns the ER as a complete organelle: When folding capacity or membrane capacity becomes limiting, an ER stress response ensues. Many types of stresses may lead to a change in the ER, including oxidative stress and NF-κB activation (117), but best characterized is the UPR (118–118b). It consists of three branches in mammalian cells, which are further intertwined downstream, leading to a fine-tuned response depending on the stress. Here, we refer to the excellent reviews (118–118b) on the UPR that appear at high frequency and limit our discussion of the topic to two remarks. The increase in ER that results from an ER stress does not necessarily lead to a change in stoichiometry between resident ER proteins. We anticipate that basic stoichiometry is constant for a particular cell type but that on top of the global increase in ER, changes of perhaps only a few proteins will lead to an ER that is more catered to the task at hand. A second issue is the question whether the stress response truly leads to a relief of stress. Typical ER stressors used in the laboratory lead to cell death; however, recent research focuses more on a physiological stress that a cell is supposed to survive. The initial stress signal leads to a response, which after some time results in the silencing of the stress signal. One would anticipate that at this time the stress is over and that the ER functions normally again, but this remains to be tested.

Redox Milieu

The factors mentioned so far in this section—folding factors, ATP, calcium, pH—all are codependent. Their regulation is intertwined with a fifth set of molecules, the redox-active ones. These are involved in and/or result from the oxidation of cysteines into disulfides during protein folding and the reduction of disulfides during the resolution of nonnative disulfides and in preparation for protein retrotranslocation into the cytosol. Disulfide exchange reactions are catalyzed by members of the PDI family. Oxidation is thought to be driven by ER oxidase 1 (Ero1), which couples the formation of a disulfide in PDI with the reduction of oxygen. The PDI family members are maintained in a predominantly reduced state, presumably owing to the high concentrations of the low-molecular-weight thiol glutathione (GSH). In the cytosol, glutathione reductase maintains a reducing environment by coupling GSH reduction to the oxidation of NADPH. No such system has been identified in the ER, with the result that the balance of reduced to oxidized GSH is much more oxidizing. It is not clear whether this is simply owing to the activity of Ero1 accepting electrons from PDI, which in turn accepts electrons from GSH. Compartmentalization of the oxidation and reduction reactions is thought to be ensured by kinetic considerations. GSH is a poor substrate for Ero1, whereas PDI is efficiently reduced by GSH. Once protein disulfides are formed, they could be reduced by GSH or PDI, but if they are solvent inaccessible, they are stabilized. Hence, the folding of the polypeptide along with the internalization of any disulfides into the structure prevents the subsequent breaking of these covalent linkages. The kinetic partitioning of the reduction and oxidation of disulfides has led to the concept that the more oxidizing GSH ratio in the ER is a consequence rather than a facilitator of disulfide formation. However, the futile reduction of exposed native disulfides by GSH would be less favored in this environment than in the cytosol, and therefore, a more oxidizing GSH ratio may be necessary for secretory protein maturation.

SUMMARY POINTS

1. Endoplasmic reticulum (ER) folding factors function as complexes assisting folding and preventing the secretion of misfolded proteins from the ER; folding factors never work alone.
2. There is a large overlap in the function of ER folding factors (functional redundancy).
3. Folding influences modification of the polypeptide chain and vice versa. Modifications include glycosylation, disulfide formation, signal peptide cleavage, and prolyl hydroxylation.
4. Proper protein folding in the ER competes with protein aggregation and degradation.
5. Protein secretion efficiency (quality control) correlates with thermodynamic stability but not with function. For exit from the ER, a protein needs to be released from folding factors and packaged into transport vesicles.
6. Manipulating the speed of folding is an activity ribosomes share with folding factors.
7. Protein folding always starts during translation, but the vast majority of folding takes place after chain termination.
8. ATP, Ca^{2+}, and proper redox balance are a condition *sine qua non* for protein folding in the ER.

FUTURE ISSUES

1. What is the structure of folding intermediates in the ER?
2. Does cotranslational folding limit the number of folding pathways a protein can take? Or, in other words, how crucial is the vectorial nature of folding in vivo?
3. What are the general rules that determine whether a protein is recognized as native instead of misfolded or unfolded? Is it determined by thermodynamic stability, kinetics, or (specific) recognition motifs?
4. Which folding factors remain to be identified? Identification of calnexin and later Ero1 changed major concepts in the ER folding field. How much more is there to come?
5. How do chaperones and folding enzymes affect folding? Do they limit the number of folding pathways? The future should allow us to delineate a complete in vivo folding pathway of a newly synthesized protein, including the mechanism of action of the folding factors and their effect on conformation.
6. Which protein modifications occur in the ER? And how precisely do the known and new ones affect folding? For instance, how abundant is phosphorylation in the ER lumen and to what extent does it affect folding?
7. Which genes are responsible for maintaining the ER milieu (ATP transporters, ionic channels) and is the ER leaky under certain circumstances?
8. A plethora of ER-related protein folding diseases will be found. Will they teach us new concepts in protein folding in the secretory pathway?

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