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Review

# Co- and Post-Translational Protein Folding in the ER

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## Abstract

The biophysical rules that govern folding of small, single-domain proteins in dilute solutions are now quite well understood. The mechanisms underlying co-translational folding of multidomain and membrane-spanning proteins in complex cellular environments are often less clear. The endoplasmic reticulum (ER) produces a plethora of membrane and secretory proteins, which must fold and assemble correctly before ER exit – if these processes fail, misfolded species accumulate in the ER or are degraded. The ER differs from other cellular organelles in terms of the physicochemical environment and the variety of ER-specific protein modifications. Here, we review chaperone-assisted co- and post-translational folding and assembly in the ER and underline the influence of protein modifications on these processes. We emphasize how method development has helped advance the field by allowing researchers to monitor the progression of folding as it occurs inside living cells, while at the same time probing the intricate relationship between protein modifications during folding.

**Keywords** chaperones, disulfide-bond formation, endoplasmic reticulum, folding enzymes, N-glycosylation, protein folding

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By nature, co-translational folding as it occurs while the protein is being synthesized on the ribosome differs significantly from post-translational folding investigated in the test tube. For instance, a higher rate of translation can influence folding either positively or negatively depending on the nature of the protein (1), and translational attenuation (ribosome stalling) at specific positions within the mRNA sequence has been found to increase the folding efficiency of multidomain proteins (2). Protein folding can initiate inside the ribosomal exit tunnel, e.g. through helix formation in the lower tunnel (3–6). Moreover, a small $Zn^{2+}$-binding domain containing 29 residues can be fully folded within the protective environment of the ribosomal exit tunnel (7). Crosstalk between this tunnel and the nascent chain has been shown to induce structural rearrangements both inside the exit tunnel and at the site of chain elongation, the peptidyltransferase center (8,9). These structural rearrangements can fine-tune the environment inside the ribosome to accommodate the divergent chemistries of different amino acid side chains, resulting in an adaptable environment to allow co-translational folding. In addition, this crosstalk can modulate elongation rate (10,11), recruitment of targeting factors [e.g. signal-recognition particle (SRP)] and translocase activity (12–15).

Co-translational translocation across the endoplasmic reticulum (ER) membrane usually requires the presence of

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an N-terminal signal sequence. Upon emergence from the ribosomal exit tunnel, the signal sequence is recognized by SRP in the cytosol (Figure 1). An important property of SRP is that it halts further translation to keep the nascent polypeptide in a translocation-competent state (16). Only after binding the SRP receptor at the ER membrane and docking of the ribosome-nascent chain (RNC) complex on the Sec61 translocon, the protein complex that constitutes the channel through which the protein is translocated across or inserted into the membrane, is translation reinitiated. The textbook knowledge described above has recently been expanded at multiple levels. Peroxisomal membrane proteins employ ER translocons to get inserted into the membrane (17) but do not have discernible ER-targeting signal peptides, and proteins without signal peptides still reach the ER in *Caenorhabditis elegans*, provided the SRP-competitor nascent polypeptide-associated complex is absent (18).

Recent electron microscopy (EM) structures of the RNC–SRP and RNC–translocon complexes (19,20) match the biochemical/biophysical evidence (3–6) that transmembrane domains can adopt a helical structure early in the ribosome and maintain it in the translocon and upon insertion into the ER membrane. Studies by Helenius and coworkers on co-translational folding of ER-targeted proteins attached to the ribosome by C-terminal extensions provide evidence that the ribosome–translocon complex and associated proteins offer a protective environment to allow early co-translational folding of the nascent chain and that the translocon can accommodate alpha-helical conformations, but not complete folding into the tertiary structure (21,22).

While the principles of co-translational folding described above have been derived in part from *in vitro* studies with purified components, they still apply *in vivo*. As recently pointed out (23), post-translational folding predominates over co-translational folding in the cell: the average half-time for folding of proteins is 30–60 min, while the translation rate in mammalian cells is approximately three to five amino acids per second and hence it takes only ~2 min to synthesize an ~50-kDa protein (24). Not only that, but ~50% of available structures for single-domain proteins show N- and C-termini in close proximity, a feature that is present in many multidomain proteins as well (Figure 2). For such proteins, folding cannot progress to completion until translation is finished and the C-terminus has exited the ribosomal tunnel.

## Making an Entry – The Role of Signal Peptide Cleavage

Once the RNC complex has been handed off to the translocon, the signal sequence is inserted into the membrane and translation continues. Signal sequences can be weak, leading to multiple locations of a protein, for instance both ER and cytosol (30,31). N-terminal modifications, such as N-myristoylation (see below), can also lead to dual targeting of proteins (for example to the ER and mitochondria) (32,33). Once docked to the ER, in most type II transmembrane proteins the signal sequence stably integrates into the lipid bilayer, forming a signal anchor, whereas nearly all secreted and many type I transmembrane proteins contain a cleavable signal peptide. Insertion of the signal sequence into the membrane is an important determinant of their topology. Signal peptide-containing proteins largely follow the 'positive-inside rule' (34) of keeping positively charged amino acids (lysine and arginine) flanking the hydrophobic stretch on the cytosolic side of the membrane. Hydrophobicity determines whether a polypeptide stretch will become a transmembrane anchor, and flanking charges determine topology. Recently, the Skach laboratory showed that for at least some signal anchors (and perhaps including many signal peptides, as they function as signal anchors until cleaved) these rules are fulfilled in sequence: the N-terminal signal peptide is inserted head first toward the ER lumen, and then an inversion in the ribosome–translocon complex is needed to acquire native topology with the N-terminus in the cytosol (13). This contrasts with current textbook knowledge that reports loop-wise insertion of signal peptides, with the N-terminus in the cytosol from the start (35). Both are bound to occur, and a single protein can end up with different topologies (30). For signal-anchor and multispanning transmembrane proteins, the eventual topology is (or multiple topologies are) determined by multiple factors including charge, hydrophobicity, lipid composition and folding of the N-terminal region, a topic that is beyond the scope of this review.

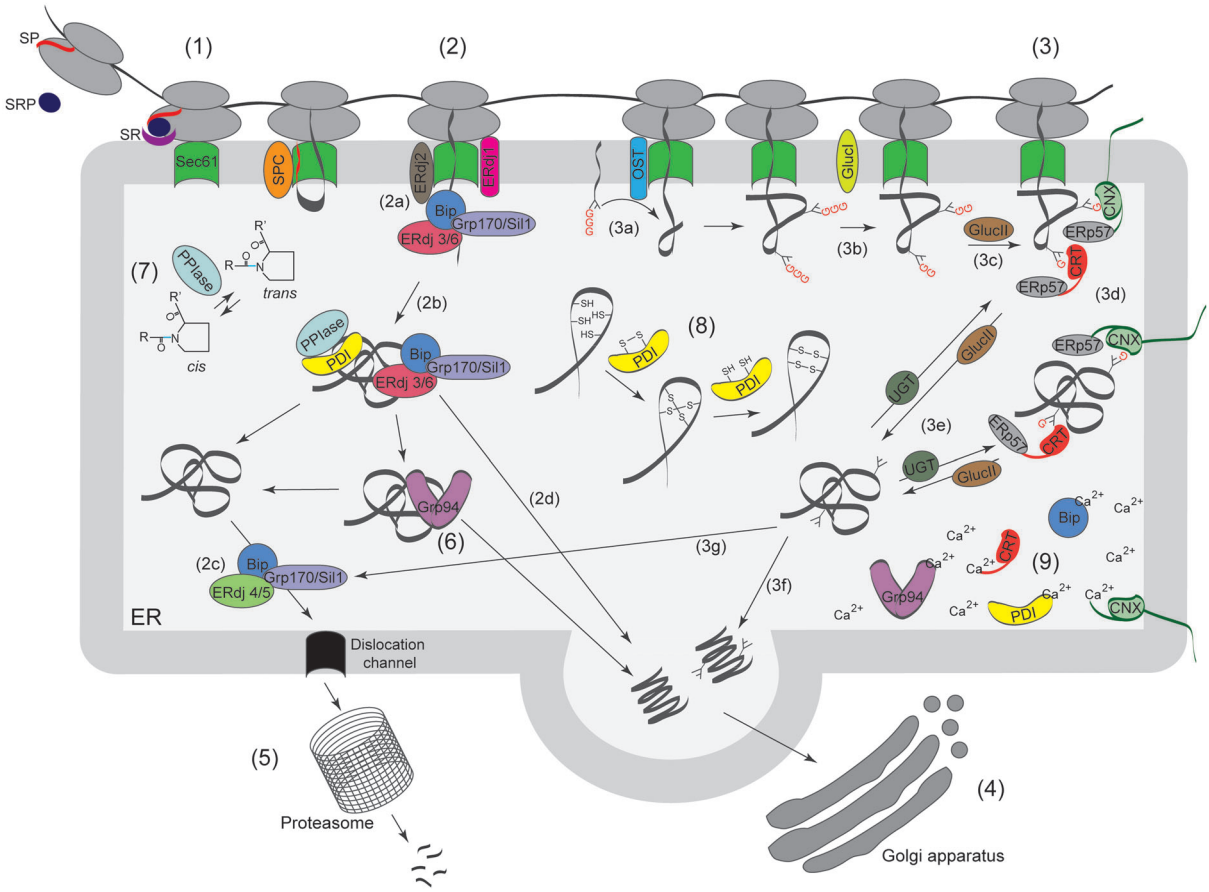


**Figure 1: Protein folding in the ER**. Newly synthesized proteins destined for the secretory pathway are targeted to the ER membrane by a signal peptide (SP), which gets cleaved by the signal–peptidase complex (SPC) (1). Signal sequences that are not cleaved serve to anchor proteins in the membrane. Once the polypeptide emerges from the Sec61 translocon, folding is assisted by chaperones and folding enzymes. Two of the main chaperone systems are BiP (2) and calnexin (CNX)/calreticulin (CRT) (3). BiP acts in concert with its co-chaperones of the ERdj family and the nucleotide-exchange factors Sil1 and Grp170, which also acts as chaperone in its own right. For recruitment to translating ribosomes and for protein translocation, BiP functions together with ERdj1 and ERdj2, respectively (2a). In protein folding, BiP cooperates with ERdj3 and ERdj6 (2b), whereas in ER-associated degradation of misfolded proteins, it collaborates with ERdj4 and ERdj5 (2c). Upon correct folding (2d), proteins exit the ER and travel to the Golgi (4), whereas misfolded proteins are dislocated to the cytosol for proteasomal degradation (5). The lectin chaperones CNX/CRT assist glycoprotein folding. N-glycosylation is initiated by the oligosaccharyltransferase (OST) complex, which transfers the $Glc_3Man_9GlcNAc_2$ core oligosaccharide from a dolichol precursor to Asn residues of Asn-X-Ser/Thr motifs present on nascent polypeptides (3a). Glucosidases I (GlucI) and II (GlucII) then trim the two outer glucoses (G) in sequence (3b, 3c) and the resulting mono-glucosylated polypeptides are recognized by CNX/CRT. Their associating co-chaperone ERp57 facilitates disulfide-bond formation in the glycosylated substrate (3d). Glycoproteins exit the CNX/CRT cycle after trimming of the last glucose residue by GlucII (3e). Correctly folded glycoproteins exit the ER (3f) and travel to the Golgi (4), whereas incompletely folded glycoproteins re-enter the CNX/CRT cycle after the readdition of one glucose residue by UDP-glucose glycoprotein glucosyltransferase (UGT) (3e). Terminally misfolded glycoproteins are directed via, e.g. BiP to a dislocation channel (3g) and the cytosol for degradation by the proteasome (5). Other important folding factors include Grp94 (6), which assists folding of proteins handed over by BiP, peptidyl-prolyl isomerases (PPIases) (7), which catalyze *cis-trans* isomerization of X-Pro peptide bonds, and protein disulfide isomerases (PDIs) (8), which catalyze the formation, reduction and isomerization of disulfide bonds. Several folding factors bind $Ca^{2+}$ (9), which is important for ER homeostasis and protein folding. R, R′: polypeptide chains.

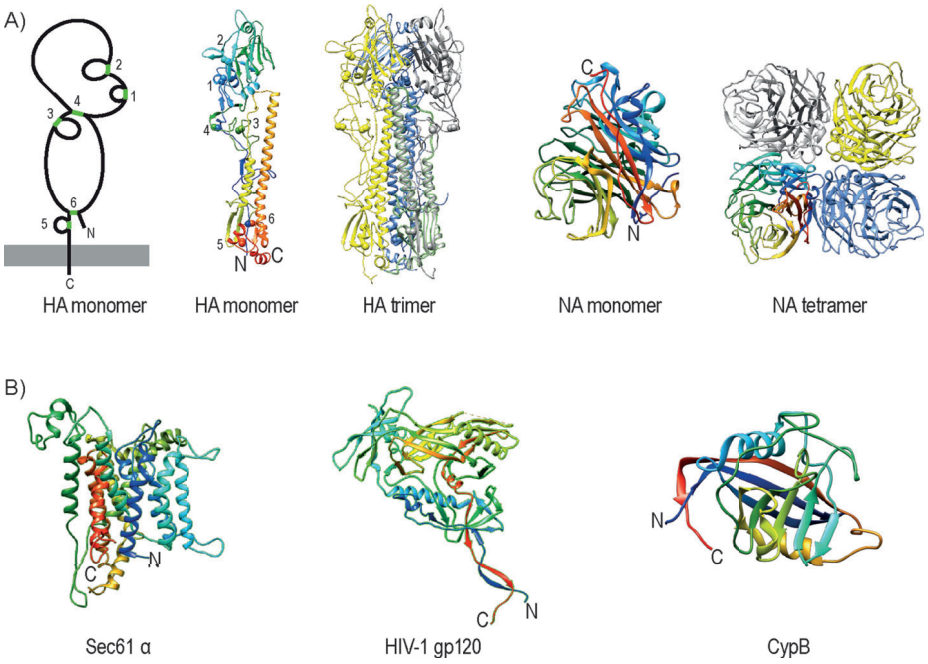


**Figure 2: Protein structures with N-C proximity**. A) From left: 'Bird' representation of influenza virus hemagglutinin (HA) drawn by Prof. Ari Helenius as in Table of Contents. Numbers indicate disulfide bonds in order of formation during folding. Crystal structures of HA ectodomain monomer (PDB: 1HA0; 25) showing N- and C-terminal contacts. HA ectodomain trimer (PDB: 1HGD; 26) with each subunit represented by a different color. NA ectodomain monomer (PDB: 4QN3; 27) and NA ectodomain tetramer (PDB: 4QN3) with each subunit represented by a different color. B) From left: Cryo-EM structure of Sec61α (PDB: 4CG6; 19), crystal structures of HIV-1 gp120 (PDB: 4TVP; 28) and cyclophilin B (CypB, PDB 3ICH; 29), which all show proximity of N- and C-termini in the folded state. HA monomer, Sec61α, gp120 and CypB structures are rainbow colored, with a blue N-terminus and a red C-terminus.

Early studies (36,37) have established that signal peptides are cleaved by the signal–peptidase complex during translation, as soon as they emerge from the translocon; the cleavage site obeys some rules concerning residues −1 and −3 (small hydrophobic), but knowledge is insufficient to predict cleavage accurately (38). This would suggest that the residues downstream of the cleavage site play no role for cleavage efficiency. However, a proline next to the cleavage site does inhibit cleavage (39,40) and residues further downstream can influence signal-peptide cleavage; for example, HIV-1 Env (41), HCMV US11 (42) and EDEM1 (43) lose their signal peptides post-translationally, effectively acting as type II signal anchors for the early period of the protein's life. Cleavage of HIV-1 Env required some folding (44), suggesting interplay between the signal peptide and the folding molecule, while delayed cleavage of EDEM1 affects its substrate specificity. These signal sequences tend to be longer than the usual 15–25 residues and likely form stable transmembrane domains (45), as does the signal sequence of Arenaviral glycoprotein C precursors (45). Although not a direct study on the role of signal-sequence cleavage on folding, the Helenius laboratory showed that a signal anchor as opposed to a cleavable signal peptide allowed complete domain folding at a shorter nascent chain length (22), suggesting that tethering of a protein's N-terminus to the ER membrane can be productive for folding.

Signal-peptide mutations have been associated with a variety of diseases including Ehlers–Danlos syndrome (46), autosomal dominant familial isolated hypoparathyroidism (47), familial central diabetes insipidus (48), factor X deficiency (49), idiopathic pulmonary fibrosis (50), familial expansive osteolysis (51) and breast cancer (52). While mutations often block SRP binding, translocation or cleavage (48,49,53), mutations in preprocollagen V(α1) (46) and IL-10 (50) signal peptides were shown to be more subtle with an equally severe outcome: they block secretion

of mutant protein despite an unchanged electrophoretic mobility, which suggests similar (but likely not identical) signal-peptide cleavage. The identity of the signal sequence clearly influences a protein's cleavage as well as its N-glycosylation (54,55), implying effects on folding. Indeed, both timing and position of signal-peptide cleavage affect folding and hence function of proteins. An alternative cause for disease-related signal peptide (cleavage) defects may lie in the non-targeting functions of signal peptides (56).

Taken together, these data suggest that in many cases signal sequences are more than simply cellular postcodes and in fact have diverse post-targeting roles in folding, maturation and function.

## Folding and Assembly of Multidomain and Membrane-Spanning Proteins

Proteins do not function alone. Their interactions range from short transient ones within functional networks to long-lived in the stable oligomers formed during biosynthesis. Many proteins in the secretory pathway are oligomeric and their assembly has been reported to occur during late folding steps or after folding, especially for homo-oligomeric proteins. Although some cytosolic proteins were found to assemble while still on the polysome (57–60), homo-oligomerization in the ER seems mostly post-translational (57). This appears counterintuitive, as the polysome limits spacing between growing nascent chains and would allow early interactions. Oligomerization has been well studied for viral glycoproteins, such as influenza virus hemagglutinin (HA) (57,61) and vesicular stomatitis virus (VSV) G protein (62,63), which both homotrimerize after or toward the end of subunit folding; HA then forms a stable, irreversible trimer (57), whereas the VSV G trimer remains in equilibrium with its (folded) monomers (64).

The vast majority of oligomeric proteins consist of different subunits for which co-translational assembly has been reported. A well-studied example is the antibody molecule (immunoglobulin, Ig), whose protomer is a heterotetramer of two light chains (L) and two heavy chains (H). IgG was the first protein for which heteromeric assembly was examined (65,66): in 1979, Bergman and Kuehl demonstrated that L assembles after completion of translation and translocation, but assembles onto H chains during their synthesis, leading to a detectable H–L assembly intermediate. For another IgG subtype the H homodimer is the dominant assembly intermediate, consistent with a co-translational assembly of H onto another H chain, most likely of 2 nascent H chains on the polysome (65,66). The assembling L chain was shown to be complete, released from the ribosome and contain at least one folded domain, but more recent work by the Hendershot laboratory has demonstrated that the assembly involves templated folding. Here, the major Hsp70 chaperone in the ER, BiP, is displaced from the unfolded H and L domains by their assembly partner (67–70). A domain that requires template-assisted folding always teams up with a folded domain that is fit to act as template.

This same theme of template-assisted folding is likely to be general, as the T-cell receptor (TCR) follows the same principle in assembly of the Ig-like domains in its alpha and beta chains (71). In this case, templating not only supports folding but could also be functional, as it may increase specificity within the T-cell repertoire (71). The generality does not stop at Ig domains as (again) the TCR alpha chain needs other TCR subunits to retain the weak transmembrane domain of the alpha chain in the ER membrane (72). In the absence of a partner subunit to assemble with, the alpha chain slips into the ER lumen, where the hydrophobic region is recognized by BiP, resulting in rapid degradation of the TCR alpha chain by the proteasome in the cytosol. For details on ER-associated degradation (ERAD), we refer to the review by Molinari and coworkers in this issue (73).

Proteasomal degradation of unassembled oligomer subunits is a common theme in the assembly of hetero-oligomers and especially for complex proteins consisting of more than two (up to even eight) different subunits. Examples are the TCR and acetylcholine receptor, but also several protein complexes required for protein biosynthesis in the ER, including the Sec61 translocon and the oligosaccharyltransferase (OST) (see below and 74–76). Formation of complex quaternary structure in the ER has been characterized well for only a dozen proteins, for some already more than 20 years ago. An insightful review on this that has withstood the test of time was published in 1989 by Hurtley and Helenius (77). In most

studied cases, the expression of a single subunit is regulated and determines the amount of oligomer formed, while the others are made in excess with orphan subunits being degraded. Regulation by more than a single subunit does occur, particularly in heterogeneous assemblies such as the TCR (72).

The atomic interactions involved in protein folding (hydrogen bonding, electrostatic and Van der Waals interactions) are identical to those involved in protein assembly/oligomerization, in transient interactions and, of course, then also identical to those involved in the assembly of domains within a multidomain protein. Most eukaryotic proteins are large and consist of more than a single domain, such as immunoglobulins, which possess several domains that fold relatively independent of each other and usually are organized as beads on a string. However, as is true for single-domain proteins (78), large proteins often have proximal N- and C-termini as well (Figure 2), which precludes vectorial folding and involves interactions of parts of the molecule that are distant in the linear amino acid sequence. Some domains may consist of a contiguous polypeptide chain, whereas other domains must integrate distant sequences. The low-density lipoprotein receptor (LDLR), despite its strictly linear domain organization, forms distant interactions during the on-pathway folding process (79). Non-native interdomain interactions during folding may well be the rule for most proteins. The structure of these proteins, such as influenza virus HA (Figure 2), with proximal ectodomain N- and C-termini and with trimerization involving the transmembrane and/or membrane-proximal domains, may well be the explanation for the counterintuitive post-translational homo-oligomerization. Even though the trimer subunits appear in close proximity as nascent chains in the ER, only after advanced folding the interaction surfaces for trimerization will have formed (Figure 2), which precludes their co-translational trimerization.

A special type of multidomain proteins are the multimembrane-spanning proteins, which during folding have domains or at least some amino acid residues in the cytosol, the ER lumen as well as in the ER membrane. These three topologies offer advantages, as compartmental separation provides a phase separation within the polypeptide sequence and prevents separated polypeptide parts from aggregating with each other. The challenge is the coordinated folding of all three protein domains, perhaps requiring concerted actions of chaperones in all three topological domains. The folding of complex multimembrane-spanning proteins, such as ABC transporters and G protein-coupled receptors (GPCRs), has barely been studied, but some intricate translocation and folding studies have been published and the many high-resolution structures do suggest common themes. Co-translationally, transmembrane segments are inserted into the ER membrane loop by loop, with downstream sequences 'pushing' N-terminal segments out of the Sec61 translocon into the lipid bilayer (15,80–82).

The Skach laboratory showed that Aquaporin1 (AQP1) is inserted co-translationally in the native topology, whereas Aquaporin4 (AQP4) needs to rearrange and even invert some of its transmembrane segments after translocation to acquire its functional conformation (83,84).

## The First Encounter – Molecular Chaperones

Once the N-terminus of a polypeptide emerges from the Sec61 channel, it starts to fold. In this process, it needs the assistance of several folding factors: the molecular chaperones and folding enzymes (Figure 1).

Molecular chaperones are defined as 'proteins that interact with, stabilize or help a non-native protein to acquire its native conformation, but are not present in the final functional structure' (85). They reside in the nucleus, cytosol, ER, mitochondria and in plants also in chloroplasts. The major ER-resident chaperones are the Hsp70 BiP (Grp78) (86), the Hsp90 Grp94 (gp96) (87) and the lectin chaperones calnexin (CNX) and calreticulin (CRT) (88), which are unique to the ER and will be discussed with N-linked glycosylation below.

BiP is the most abundant and most versatile of ER chaperones. It contributes to protein folding (89), is always present in misfolded protein aggregates and retains misfolded and unfolded proteins in the ER (90–93), assists retrograde transport of proteins destined for proteasomal degradation (94) and regulates the unfolded protein response (95). BiP helps maintain $Ca^{2+}$ homeostasis (70,96), which indirectly is crucial for protein folding as most ER-resident

folding factors bind abundant $Ca^{2+}$ and depend on it for function (Figure 1). Calcium levels directly affect folding of $Ca^{2+}$-binding proteins, such as integrins (97,98) and LDL-A and EGF repeat-containing proteins including the LDLR and LRP (99,100). Fitting with BiP's role as master regulator of ER function (86), its deletion does not generate viable cells or mice: BiP-knockout mice show peri-implantation lethality (101). Inducible inactivation by subtilase cytotoxin (102) or BiP depletion has confirmed the importance of BiP (103).

Client binding by BiP is regulated by its ATPase cycle through the actions of J proteins (ERdj1–7) (104) and nucleotide-exchange factors (Grp170, Bap/Sil1) (105,106) (Figure 1). BiP is the chaperone that an emerging nascent chain is likely to encounter first, before any other chaperone, because one of the J proteins that recruit BiP and activate its ATPase activity, ERdj2/Sec63, is a component of the translocon. CNX competes for binding of glycoproteins (see below), but even then BiP may facilitate translocation (107,108).

While BiP binds perhaps even all proteins that pass through the ER, its best-characterized role is in folding and assembling antibody molecules. Haas and Wabl identified BiP as Ig-binding chaperone (109), and the Hendershot laboratory showed that BiP holds disordered Ig domains in antibody H and L chains as well as in the TCR until handing over to the assembly partner (68,70,72). Assembly with the stable partner-Ig domain induces folding of the disordered domain into a stable native oligomer, which involves a trapped folding intermediate that requires the proline-isomerase CypB to progress to the functional fold (110). Studies like this that clarify the molecular effect of a chaperone on a folding protein in the ER still are rare, even though most ER-resident folding factors have been shown to be essential for a plethora of processes and phenotypes.

The ERdJs, as co-chaperones for BiP, recruit BiP to specific processes, such as translocation (111,112), folding (113–115) and degradation (116–118), and may bind clients first and independent of BiP before presenting them to the chaperone. Although reported crucial for folding, there is no evidence (yet) that any of the J proteins affect protein folding directly and independently of BiP (Figure 1). Moreover, there is no evidence (yet) that BiP itself directly affects folding of a protein, meaning that it would direct folding and change substrate conformation. Like other Hsp70s it is thought to be a rather passive chaperone that holds and thereby on the one hand prevents misfolding and aggregation and on the other hand maintains folding competence in the client protein (71,86,119).

The other major ER-resident chaperone of a general family is the Hsp90 Grp94, which caters to a more limited clientele (120,121), including Toll-like receptors (TLRs), integrins (122–125), immunoglobulins (126,127), collagen (128), insulin-like growth factors (129,130) and members of the LDLR family (121,123). Although ATP binding and hydrolysis are essential steps for Grp94 chaperone activity (131), the exact mechanism of action of Grp94 has not been elucidated. Recently, only a single putative co-chaperone family of Grp94 has been identified, the Canopy (CNPY) family (132), including the TLR-specific CNPY3/PRAT4A (133) and CNPY5 (Mzb1/pERp1) (133–136).

Hsp90s usually bind substrates after Hsp70s and indeed, BiP hands over newly synthesized immunoglobulin to Grp94 (137) (Figure 1). How Grp94 affects folding is not clear, but it was shown to be essential in many processes in the ER, including protein folding (121), ER quality control and stress response (138) as well as $Ca^{2+}$ buffering (139).

Next to the abundant general and ER-specific molecular chaperones, the ER contains various classes of so-called folding enzymes, which catalyze co- and post-translational modifications important for protein folding. A special class of folding enzymes are the peptidyl-prolyl *cis-trans* isomerases (PPIases), which catalyze the isomerization of the peptide bond preceding proline residues (prolyl bonds) from *trans* to *cis* orientation and back (Figure 1). The rotation of the prolyl bond has a very high activation energy (140) resulting in extremely slow isomerization reactions with time constants of 10–100 seconds at 25°C. As such, proline isomerization often is a major rate-limiting step during protein folding, unfolding and refolding (141–147). This is obvious considering that almost every protein contains proline residues (148). Prolines are added by the ribosome in *trans* (149,150) and multiple *cis-trans* isomerizations may be needed during folding (29,143,151,152). In protein structures 5–7% of prolyl bonds are in *cis* (153–155), with structural analyses showing almost half

(43%) of PDB structures containing at least one *cis* prolyl bond. *In vitro* refolding studies on disulfide-containing proteins, such as ribonuclease (142), conotoxins (156) and minicollagen-1 (157), have revealed that slow proline isomerization can be rate limiting for oxidation and reduction of disulfide bonds, which can be accelerated by PPIases. PPIases clearly are important and general components of the folding machinery that may well cover all folding proteins as their substrates.

It is therefore surprising that different classes of PPIases exist: the cyclophilins (Cyps) (158), which are inhibited by cyclosporin A, the FKBPs, which are inhibited by FK506, and the parvulins, which are found to work on folded proteins (159–161). Functional studies *in vitro* have shown that prolyl bonds can be isomerized by either Cyp or FKBP family enzymes with kinetics shown to be dependent upon neighboring residues (162–164). CypB-dependent isomerization of a single proline residue controls disulfide-linked assembly and secretion of IgG (165, see above), and CypB directly catalyzes triple-helix formation of type III collagen (143, see below).

To what extent PPIases exhibit substrate specificity *in vivo* has yet to be tested extensively. Such specificity may be provided by the networking with other ER-resident folding factors, as multiple PPIase-containing protein complexes have been found that include BiP, Grp94, oxidoreductases, CRT and CNX (152). In fact, the entire ER folding machinery consists of networks of chaperones and folding enzymes, which act on substrates together, either simultaneously or in sequence (152,166,167) (Figure 1).

## The Role of Protein Modifications

Protein modifications, often occurring co-translationally, play an integral and crucial function in ER protein folding. This is especially the case for the addition of disulfide bonds and N-glycans, but other modifications specific to the ER also affect the folding process. Importantly, the development of methods to monitor the introduction of such modifications co-translationally in living cells has been critical. Several such methods were pioneered in the Helenius laboratory (see Box 1), and have enabled us to understand at a quite detailed level how proteins fold inside the ER of living cells.

### Box 1. Techniques for Studying Co-Translational Folding in the ER

Recent years have seen the application of sophisticated biophysical techniques, such as NMR spectroscopy (168,169), mass spectrometry (170) and electron microscopy (3,7) to study co-translational folding. While electron microscopy has been instrumental in generating structural insights into the complex between ribosome, nascent chain and translocon (19,20,171), given the complex environment of the ER lumen, such techniques are not likely to become applicable to ER protein folding in the near future. Here, we provide an overview of the most commonly used methods to study co-translational protein folding in the ER.

Pulse-chase approaches to study protein folding as pioneered by Helenius and coworkers involve metabolic radiolabeling of newly synthesizing proteins (24,61,172), which allows folding to be studied with time. By combining radiolabeling with immunoprecipitation and both non-reducing and reducing SDS–PAGE, oxidative folding can be visualized as electrophoretic mobility changes caused by changes in the compactness of the SDS-denatured protein due to disulfide bonding. As an alternative, radiolabeled amino acids can be incorporated by *in vitro* translations, whereby only the mRNA of the protein of interest is translated. Ribosomes and translation components are supplied by a cellular extract, normally rabbit reticulocyte or wheat germ lysate, and these systems are supplemented with ER-derived membranes (semi-permeabilized cells; 173, 174) or ER-derived microsomes (175–177) to study co-translocational processes of membrane and secretory proteins.

By using short pulse times in relation to a protein's synthesis time and compounds (e.g. cycloheximide) that inhibit chain elongation, folding differences can be probed even of nascent chains still attached to the ribosome. Analysis of nascent chains has been taken to the next level by the use of 'diagonal' 2D electrophoresis where the first dimension is non-reducing and the second dimension is reducing SDS–PAGE.

By comparing positions of bands relative to the diagonal, intrachain as well as interchain disulfide bonds are distinguished, and it is possible to estimate how extended a nascent chain must become before it attains disulfides or interacts with chaperones and oxidoreductases (178). Co-translational folding can also be studied on ribosome-arrested chains of different lengths. Removal of the stop codon from mRNA translated *in vitro* prevents termination of the polypeptide chain. Folding of the ribosome-arrested chain can then be followed, for instance to determine the minimum distance from the ribosomal peptidyltransferase center needed for domain folding, glycosylation and disulfide-bond formation (22,55).

In the case of proteins without disulfide bonds, which will not display a mobility shift on SDS–PAGE gels when comparing reducing and non-reducing conditions, limited proteolysis is a tried and proven approach. It works on the principle that folded domains are less accessible to proteases than proteins in a more extended conformation. Limited proteolysis therefore will provide global information on protein stability as well as detailed information on protein folding and assembly (179,180).

An elegant additional method to study protein folding is the use of conformation-specific antibodies. During the folding process epitopes will form or become shielded rendering them inaccessible. Using antibodies specific for different folded states in combination with broadly recognizing antibodies allows visualization of folding intermediates by SDS–PAGE (24). Antibodies directed against specific domains/regions/peptides are useful in particular when used in conjunction with limited proteolysis to identify protease-resistant domains.

As N-linked glycosylation plays such a large role in protein folding in the ER, it is also often examined to provide information about the folding state of proteins. When N-glycans become modified in the Golgi, they become resistant to digestion by endoglycosidase H (endo H), but remain sensitive to N-glycanase F (PNGase F). This difference is used to identify the cellular localization of proteins during biosynthesis (181). Specific manipulation of cellular exoglycosidases, such as α-mannosidases or glucosidases combined with the above approaches, identifies the roles of specific glycan structures. The Helenius laboratory used these approaches to delineate the glycan regulation of the calnexin cycle (76,94,182).

## Disulfide-Bond Formation

Disulfide bonds are introduced into most proteins synthesized in the ER and primarily function to indirectly stabilize the native protein structure and oligomeric complexes. They play important functions during folding (183,184), and missense mutations that replace a cysteine in a disulfide (or introduce an additional cysteine into the protein) frequently are detrimental for function, proper folding or protein stability. As a consequence, such mutations often are disease causing, as in the case of insulin where cysteine mutations can lead to diabetes (185).

Treatment of cells with dithiothreitol (DTT), a cell-permeable reducing agent, has been particularly useful in investigating the importance of disulfide-bond formation for protein folding in live cells, as pioneered in the Helenius laboratory (186–188). Such studies have made it clear that disulfide-bond formation often is essential for folding and that for many proteins it occurs already co-translationally. Various fully synthesized proteins that have been kept reduced, however, can also fold to the native conformation upon removal of DTT (186,189). Influenza virus HA has even been established to fold to completion under *in vitro* conditions upon formation of a few specific disulfide bonds in the intact ER (190). This fits well with the finding that even conserved disulfides can be dispensable for folding and function, while others are essential (191).

Disulfide-bond formation starts co-translationally, with post-translational reshuffling (isomerization) occurring in what is often the rate-limiting step in folding (192,193). While certain proteins, such as influenza HA, go through defined folding steps characterized by folding intermediates containing native long-range disulfide bonds (24,178), slow-folding proteins such as HIV-1 envelope glycoproteins initially form disulfide bonds that then need to undergo extensive isomerization before the native conformation is reached (44). In the case of the LDLR, long-range

non-native disulfide bonds even form abundantly during on-pathway folding to the native state (79). For this multidomain protein, folding therefore does not proceed in a vectorial domain-by-domain order, but rather through isomerization of commonly occurring intradomain non-native disulfides. As discussed above, many proteins have proximal N- and C-termini, which may suggest that peripheral contacts are favorable for efficient folding, perhaps by shielding hydrophobic sites, reminiscent of the hydrophobic collapse observed during *in vitro* refolding of unfolded proteins (194). Time will tell whether other proteins that are extended in their functional conformation are using a similar pathway toward the native state.

Disulfide-bond formation, reduction and isomerization are catalyzed by thiol-disulfide oxidoreductases of the PDI family (Figure 1). Comprising around 20 human members (195), these enzymes play important roles during folding (and degradation) of many proteins. PDIs contain a Cys-Xaa-Xaa-Cys active-site motif embedded in domains with a thioredoxin-like fold (196). In the oxidized state, the active site catalyzes substrate oxidation by disulfide exchange, i.e. the active-site disulfide is simply transferred to the substrate. In the reduced state, PDIs catalyze reduction and isomerization (197). The latter type of reaction may also occur through consecutive rounds of reduction and oxidation (198).

During disulfide exchange, a mixed-disulfide intermediate forms between enzyme and substrate (183). These transient species are inherently difficult to trap in living cells. In a seminal study from the Helenius laboratory, it was demonstrated that PDI and its close homologue, ERp57, co-translationally form mixed-disulfide complexes with Semliki Forest virus (SFV) glycoproteins (199). This study was the first to trap PDI family members with growing nascent chains in the process of oxidative folding. Moreover, the data indicated that both PDI and ERp57 also formed mixed disulfides with the full-length SFV glycoproteins and catalyzed disulfide isomerization in their substrates. Finally, this work demonstrated that ERp57 and SFV glycoproteins existed in a ternary complex with the lectin chaperones CNX and CRT (see below and 76), underscoring the function of ERp57 as a co-chaperone for CNX and CRT especially dedicated to glycoprotein folding (200) (Figure 1). The fact that cells devoid of ERp57 can still fold SFV glycoproteins efficiently was found to rely on replacement of ERp57 with ERp72 (another PDI family member) (172), indicating redundancy among ER oxidoreductases (201).

Upon substrate oxidation, active-site cysteines are left in the reduced state and must be recycled to the disulfide state. Oxidation of PDI by the Ero1 oxidase constitutes the most important reoxidation pathway, but in recent years it has become clear that several additional such pathways exist (202). For instance, $H_2O_2$ can support oxidative folding facilitated by peroxiredoxin 4 in cells devoid of Ero1 (203). While PDI is the primary cellular substrate of Ero1 (204), it has been shown that PDI family members can exchange disulfides among each other (204,205), abolishing a requirement for all enzymes to become directly oxidized by Ero1 (or reduced by a potential ER-localized reductase, as discussed below).

Given the importance of disulfide isomerization for correct folding, it is crucial for the cell to also ensure that a fraction of active-site cysteines in PDIs are maintained in the reduced state. For instance, recent work has demonstrated that the PDI family member ERdj5 (which is also a J protein that stimulates BiP's ATPase activity) functions to reduce non-native disulfides during folding of the LDLR (206). The exact mechanism by which active-site cysteines in PDIs are kept in the reduced state is unknown, but glutathione likely plays a role in balancing ER redox conditions through reaction with the active sites of PDI family members (207). In addition, an as yet undiscovered reductive pathway coupled to NADPH as the electron donor could also exist (208). In another recent exciting development in this field, it was shown that oxidative folding of, e.g. LDLR proceeds through two phases with differential requirements for oxygen (209). In the initial rapid formation of disulfide bonds, oxygen was shown to be dispensable, whereas post-translational isomerization and dithiol oxidation were dependent on oxygen. These findings indicate that electron acceptors other than molecular oxygen can support oxidative folding in the initial phase of disulfide-bond formation.

## N-Linked Glycosylation

N-glycans are added to the asparagine residue in a simple acceptor sequence (the 'sequon'), which is typically

Asn-Xaa-Ser/Thr (where Xaa can be any amino acid residue except proline). The $Glc_3Man_9GlcNAc_2$ core oligosaccharide (76) contains three branches, and is transferred from a lipid-linked precursor onto the polypeptide chain by the oligosaccharyltransferase. This enzyme complex essentially comes in two flavors: one contains the catalytic STT3A subunit and is associated with the translocon to perform co-translational glycosylation, whereas the other – containing the STT3B isoform – glycosylates acceptor sites skipped by STT3A (e.g. to carry out post-translational glycosylation) (210).

The addition of N-linked glycans plays a key role in ER protein folding (211), mainly through their interaction with the lectin chaperones CNX and CRT, which positively affects glycoprotein folding efficiency (Figure 1). However, N-glycans also possess intrinsic physicochemical properties that accelerate folding, enhance thermodynamic stability and decrease aggregation propensity of proteins (reviewed in 212).

As detailed elsewhere in this issue by Hebert and coworkers (76), the homologous CNX (membrane-bound) and CRT (soluble) bind mono-glucosylated ($Glc_1Man_9GlcNAc_2$) trimming intermediates of the core glycan to assist co- and post-translational folding of a large variety of N-glycosylated proteins (88). Upon release from CNX/CRT, substrates can exit the ER if they are correctly folded. Alternatively, they rebind CNX and/or CRT, which allows them another chance to fold while ensuring ER retention and prevention of aggregation. In this so-called CNX/CRT cycle, binding to and release from CNX and CRT are controlled by independently acting enzymes that modify the N-glycan structure. While bound by either lectin chaperone, the substrate glycoprotein is exposed to the associated co-chaperone ERp57 that facilitates disulfide-bond formation (213). Terminally misfolded glycoproteins are degraded by the ERAD pathway in a process that is also controlled by the structure of the N-linked glycan (73). Overall, CNX and CRT binding slows down glycoprotein folding kinetics, prevents premature ER exit and increases folding efficiency.

Recently, another ER-resident lectin was identified, malectin, which binds to terminally glucosylated oligosaccharides *in vitro* (214,215), leading to the hypothesis that it may participate in the CNX/CRT cycle. Molinari and coworkers demonstrated, however, that malectin exhibits different substrate-binding affinities and kinetics than CNX, with a preference for misfolded conformers of influenza virus HA (216). This, and the findings that malectin expression is ER-stress sensitive, and its overexpression interferes with protein secretion, points to a putative role in binding and retention of misfolded glycoproteins in the ER (216).

The importance of N-glycans for glycoprotein folding, assembly and secretion is well documented from numerous investigations (217). Given the many positive effects of N-glycosylation for folding and stability, it can be difficult to determine exactly the underlying mechanism of deleterious effects of deleting N-glycosylation sites in specific proteins. However, chemical inhibitors of glycoprotein interaction with CNX and CRT exist, which completely inactivate the CNX/CRT cycle, while maintaining the N-glycan. In addition, knockout cell lines are available for each protein (218–220).

Here, we will draw attention to select examples of how N-glycosylation influences protein folding and assembly to illustrate the versatility of this modification. It is clear that the position of the N-glycan can determine chaperone selection during folding (55). In an important study from the Helenius laboratory, it was shown that the position of the first N-glycan in the polypeptide chain governs the preferred nascent chain interactions with CNX and/or CRT over BiP (221). Overall, the data indicated that the presence of an N-glycan within the first 50 amino acid residues dictates interaction with CNX/CRT, while BiP first binds the nascent chain in the absence of such N-terminally placed N-glycans. On the other hand, the role of BiP during translocation may still allow an even earlier transient interaction with BiP. When employing inhibitors of the CNX/CRT interaction, glycoproteins normally interacting with CNX and/or CRT instead will bind BiP, demonstrating redundancy among the two chaperone systems (195,222–224). A similar finding has recently been published for a disease-causing mutant of fibulin-3, in which N-glycosylation can 'mask' a binding site for Grp94 that is first efficiently exposed when an N-glycan important for CNX/CRT interaction is removed (225).

This work also demonstrated that mutation of a particular N-glycan important for CNX/CRT interaction selectively conferred negative effects, e.g. increased cellular aggregation and degradation, to the disease-causing mutant when compared with the wild-type protein.

It has also become apparent that N-glycans present on the same polypeptide chain are not equivalent. Thus, mutational analysis has shown that individual glycans differentially influence the rates and efficiencies of folding, oligomerization and secretion; CNX versus CRT binding; and cellular stability (226–234). Importantly, as detailed in the following section, N-glycosylation also critically influences folding through its close connection with disulfide-bond formation.

## Interplay Between N-Glycosylation and Disulfide-Bond Formation

As N-glycosylation and disulfide-bond formation often occur co-translationally, and since many proteins comprise a number of both these modifications, it is only natural that these two processes are intricately connected during folding (235). These connections have been elegantly explored in the Helenius laboratory using a pulse-chase/immunoprecipitation approach in combination with 2D SDS–PAGE (178). As detailed in Box 1, this method can monitor both co- and post-translational N-glycosylation and disulfide-bond formation. When applied to study influenza virus HA folding, the results showed that the formation of disulfide bonds was not a strictly coordinated event in all polypeptide chains, as specific disulfides would form either during or after complete translation of the protein (178). Moreover, it also became clear that inhibitors of N-glycosylation or CNX/CRT interaction perturbed proper disulfide-bond formation. Indeed, when deprived of the interaction with ERp57, certain glycoproteins fail to form correct disulfide bonds (172,236), and from mutational studies it is clear that removal of sites for N-glycosylation can perturb disulfide-bond formation (230–232). Individual N-glycans have been suggested to be positioned close to cysteine residues that need to shield – through interaction of the nearby glycan with CNX or CRT – from non-productive (intermolecular) disulfide-bond formation (55).

The majority of sites for N-glycan addition are present in predicted flexible regions, such as loops (237). At the same time, an extended and non-restricted conformation of the polypeptide chain is preferred by OST (238). Therefore, these quite recent data predict that protein folding could compete with N-glycosylation. Indeed, previous studies show this to be the case. Investigations in *Saccharomyces cerevisiae* using Carboxypeptidase Y and in a cell-free system as well as mammalian cells using tissue-type plasminogen activator have collectively shown that conditions that favor folding result in less efficient glycosylation (239–241). Similarly, in cells with a truncated core N-glycan, glycosylation is slow due to lower OST affinity and proteins are hypoglycosylated (242). The same studies show that conditions that prevent folding – e.g. in the presence of a reducing agent to counteract disulfide-bond formation – lead to increased glycosylation at sequons only partially utilized under normal conditions. In general, when perturbing either of the two processes, N-glycosylation or disulfide-bond formation, the other is likely influenced as well.

The intimate connection between N-glycosylation and disulfide-bond formation is underscored by the finding that two subunits of the OST, the paralogs MagT1 and TUSC3, are thiol-disulfide oxidoreductases required for efficient N-glycosylation of acceptor sites located close to cysteine residues (e.g. NCS/T sequons or sites bracketed by a disulfide bond). The function of these two proteins was established initially for the yeast orthologs Ost3p and Ost6p (243), and supported by structural and biochemical studies on TUSC3 (74). These investigations have shown that the two proteins localize to the ER membrane and contain a thioredoxin-like domain with an embedded typical Cys-Xaa-Xaa-Cys active-site motif. The reduction potential of the active-site disulfide is highly reducing, showing that the two cysteines prefer the oxidized state, as confirmed for MagT1 (244). Based on the biochemical and structural work, a model was suggested whereby MagT1/TUSC3 (Ost3p/Ost6p) forms a transient mixed disulfide with certain glycoproteins (74,243). The mixed disulfide arises as a result of a nucleophilic attack by a cysteine thiolate from the glycoprotein on the active-site disulfide of the oxidoreductase. This intermolecular covalent interaction is proposed to stall glycoprotein folding by delaying disulfide-bond formation, which

in turn will increase glycosylation efficiency on nearby sequons.

This model was recently corroborated and extended to identify the types of substrates preferred by MagT1 in HeLa cells, which do not express TUSC3 (244). Here, it was shown that MagT1 associates only with OST complexes containing the STT3B isoform, and thus is involved in glycosylation of substrates skipped by STT3A during translation. Based on the analysis of a variety of acceptor sites, the main class of substrates for MagT1-dependent glycosylation was proposed to be those containing cysteines involved in disulfide-bond formation flanking the acceptor site, as in, for example, factor VII (244). In essence, the slowdown of protein folding resulting from MagT1 binding to substrate glycoproteins increases the glycosylation efficiency, a finding completely in line with the results demonstrating competition between folding and glycosylation.

## Other Protein Modifications

While the protein modifications mentioned above are crucial for folding of most proteins synthesized in the ER, other modifications are directed toward a smaller subset of proteins, or are less well understood.

Fatty acylation occurs in the cytosol, on both cytosolic proteins and the cytosolic tails/domains of transmembrane proteins, and comes in two main flavors: N-myristoylation and S-acylation.

N-myristoylation is the attachment of myristate, a 14-carbon saturated fatty acid to the N-terminal glycine of certain eukaryotic proteins. Although normally considered to be a post-translational modification, myristoylation occurs co-translationally after removal of the leader methionine to expose an N-terminal glycine (245,246). While myristoylation has been characterized mainly as a modification of cytoplasmic proteins, viral membrane proteins can be N-myristoylated as well: for the large envelope protein of hepatitis B virus (HBV L), a polytopic membrane protein, myristoylation is essential for viral infectivity and intracellular retention (247,248). This permanent modification may tether the N-terminus of a protein to the membrane, which is likely to affect its folding during translation.

S-acylation of proteins is the post-translational attachment of long-chain fatty acids, mostly palmitate, to cysteine residues. Palmitoylation is unique among lipid modifications because it is a reversible modification that allows it to play a role in regulation and fine-tuning of protein function similar to ubiquitination and phosphorylation (249). Palmitoylation also occurs on the cytoplasmic tails or domains of transmembrane proteins, such as GPCRs (250), intramembrane proteases (251,252), PDI family members (253) and chaperones (253,254). CFTR (255), influenza virus HA (256) and LRP6 (257) need palmitoylation to leave the ER. Especially for multimembrane-spanning proteins, such as ion channels with less hydrophobic transmembrane helices, fatty acylation may well be essential for folding and domain assembly to the native, functional conformation.

A recent review (258) pointed out that the cellular and functional consequences of palmitoylation are not necessarily direct when it comes to protein folding, but rather caused by processes such as conformational changes of transmembrane domains, regulation of membrane domain association and protein complex formation. Recent examples from the literature include the importance of palmitoylation for inositol 1,4,5-trisphosphate receptor (IP3R) function (259), which in turn influences ER $Ca^{2+}$ flux and thereby ER protein folding, and for modulation of ER subdomain localization of the transmembrane PDI family member TMX (253). Moreover, the palmitoylation status of CNX can determine its association with the ribosome–translocon complex (260) and SERCA2b (254), which directly influences glycoprotein folding and ER $Ca^{2+}$ content, respectively. These examples demonstrate that regulation of ER protein folding can be a secondary consequence of modification of the protein folding machinery. Whether this excludes a direct effect on folding remains an open question, as the processes mentioned above all involve conformational changes, which by definition influence folding.

Proline hydroxylation has long been known and recognized to be important for protein folding and stability in the ER. At the same time, the primary substrate of proline hydroxylation, procollagen, is a 'showcase' for the interdependence between post-translational modification and protein folding. Notably, procollagen folding is assisted by

more than 20 chaperones, folding enzymes and enzymes that catalyze procollagen modification (261).

Procollagen is extensively proline hydroxylated, which influences the conformation of the polypeptide backbone to allow more efficient triple-helix formation. At the same time, proline hydroxylation has been shown *in vitro* to be essential for stability of the triple helix at physiological temperatures (262). A prerequisite for formation of the triple-helix structure is the isomerization of all prolines to the *trans* conformation (263,264). Given that certain prolyl hydroxylases and PPIases are found in complex (265), the modifications catalyzed by these types of enzymes likely work in concert to generate thermostable procollagen in cells (261). The exact role *in vivo* of the PPIases CypB and FKBP65 during collagen biosynthesis is unclear, as both are found in complex with other factors crucial for collagen folding. CypB has been identified in complex with prolyl 3-hydroxylase 1 and cartilage-associated protein (261) and as a heterodimer with lysine hydroxylase 1 (261). Upon triple-helix formation, the properly folded procollagen molecule is no longer a substrate for prolyl (and lysyl) hydroxylation (261). At this point, the collagen-specific chaperone Hsp47 binds procollagen to prevent its premature association into bundles and to promote its ER-to-Golgi transport (266).

Prolyl hydroxylase is known to require ascorbate (vitamin C), and scurvy leads to misfolding and ER retention of procollagens. Recent evidence provides an unexpected link between proline hydroxylation and oxidative protein folding. Thus, conditions that lead to excess production of $H_2O_2$ in the ER were found to deplete lumenal ascorbate by oxidative inactivation, which in turn damaged proline hydroxylation of procollagen and led to an unconventional type of scurvy (267,268).

In addition to the known substrates (pro)collagens, the collagen-like adiponectins (269), conotoxins (270) and some cell-wall proteins in plants, other substrates for prolyl hydroxylases are bound to be identified. The same is true for lysine hydroxylation, which has been studied sparsely and is also connected to procollagen and conotoxin biosynthesis. Interesting is the secondary modification of hydroxylysine, which can be glycosylated thereby changing the character of the nearby (collagen) polypeptide chain even more (271,272).

Unlike proline and lysine hydroxylation, acetylation of lysine has only recently been found in the ER (273). The modification occurs on several ER-transiting and ER-resident proteins, including chaperones and enzymes involved in protein modification and folding (274), and two involved acetyltransferases are known (275). The exact function of lysine acetylation is still not clear, but it may well serve distinct purposes for ER-transiting versus ER-resident proteins. Lysine acetylation in newly synthesized proteins seems to take place on correctly folded proteins with non-acetylated species being subject to degradation in a post-ER compartment by the PCSK9 protease (276), whereas modification of ER-resident proteins has been speculated to influence their function and/or activity (277). Disruption of ER lysine acetylation leads to cell death and is associated with serious human diseases (277,278). Many questions concerning lysine acetylation in the ER remain unanswered (277), e.g. how acetyltransferases distinguish substrates from non-substrates, and it will be intriguing to follow this topic in the future.

## Concluding Remarks

With about a third of all human proteins being synthesized in the ER, correct protein biogenesis in this organelle is of crucial importance to cellular and organismal function. As illustrated in this review, ER protein folding and assembly are carefully orchestrated processes that most often require the interplay between several chaperones and folding factors as well as different kinds of co- and post-translational modifications. Such modifications contribute directly to increased protein solubility and stability, but also serve important purposes in directing the folding process by recruitment of chaperones. Considering the complexity involved in folding and assembly of, e.g. procollagen or hetero-oligomeric complexes of membrane-spanning proteins each containing a variety of disulfide bonds and N-glycans, and the many potential pitfalls encountered during this process, it is astounding that these processes work quite efficiently for many proteins.

The newly synthesized protein will dictate and direct some of these processes because during folding, driven by its

own internal forces, the protein will transiently expose and shield modification sites as well as chaperone and folding-enzyme interaction sites. External regulation is imposed by the collective network of molecular folding factors, local activity of which is controlled by co-chaperones, chaperone modifications as well as the folding protein, as it for instance stimulates ATP hydrolysis in BiP. Overall, differential regulation of components of the chaperone network, such as through ER stress responses, at the transcriptional, translational and post-translational levels aims to produce, for each cell at any point in time, the optimal set of chaperones, folding enzymes and folding conditions in the ER that ensure a healthy life.

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