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# Protein folding includes oligomerization – examples from the endoplasmic reticulum and cytosol

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A correct three-dimensional structure is a prerequisite for protein functionality, and therefore for life. Thus, it is not surprising that our cells are packed with proteins that assist protein folding, the process in which the native three-dimensional structure is formed. In general, plasma membrane and secreted proteins, as well as those residing in compartments along the endocytic and exocytic pathways, fold and oligomerize in the endoplasmic reticulum. The proteins residing in the endoplasmic reticulum are specialized in the folding of this subset of proteins, which renders this compartment a protein-folding factory. This review focuses on protein folding in the endoplasmic reticulum, and discusses the challenge of oligomer formation in the endoplasmic reticulum as well as the cytosol.

## What is protein folding?

During translation, amino acids are coupled via peptide bonds to create a linear polypeptide chain. This chain adopts an energetically favorable conformation during which hydrophobic amino acids are buried on the inside of soluble proteins and hydrophilic residues are mostly found in solvent-accessible sites. During the formation of the native structure, stabilizing hydrogen bonds, electrostatic and van der Waals' interactions and, in some cases, covalent bonds are formed. The formation of native secondary and tertiary structure is called protein folding, whereas the formation of quaternary structure is referred to as oligomerization

**Abbreviations**
AHSP, α-hemoglobin stabilizing protein; ATF, activating transcription factor; BAP, BiP-associated protein; CAD, caspase-activated DNase; CH, heavy chain constant domain; CL, light chain constant domain; COPI/II, coat protein complex I/II; CypB, cyclophilin B; EDEM, endoplasmic reticulum degradation-enhancing α-mannosidase-like; eIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; Ero1, endoplasmic reticulum oxidoreductin 1; GRP, glucose-regulated protein; Hsc, heat shock cognate; Hsp, heat shock protein; ICAD, inhibitor of caspase-activated DNase; Ire1, inositol requiring protein 1; LDL, low-density lipoprotein; MHC, major histocompatibility complex; PDI, protein disulfide isomerase; PERK, PKR-like endoplasmic reticulum kinase; PPIase, prolyl-peptidyl isomerase; RNAP, RNA polymerase; SRP, signal recognition particle; TCR, T-cell receptor; Tg, thyroglobulin; TRAP, T-cell receptor-associated protein; UGGT, UDP-glucose:glycoprotein glucosyltransferase; UPR, unfolded protein response; VH, heavy chain variable domain; VL, light chain variable domain; XBP1, X-box binding protein 1.

 

or assembly, although this process is in fact an extension of and includes protein folding. The distinction between an oligomer and a protein complex is unclear. Hurtley and Helenius [1] provided useful operational criteria that still apply: the main criterion is that, in an oligomer, the subunits are permanently associated and are handled and degraded by the cell as a unit, whereas protein complexes or assemblies are more dynamic.

In the early 1960s, Anfinsen *et al.* [2] showed that the information required to form a native structure is contained in the amino acid sequence itself. According to Levinthal's paradox, it is impossible for proteins to sample all possible conformations to find that which is most stable [3–5]. This led to the concept of funnel-like energy landscapes [6], according to which proteins can follow multiple routes to the native state. Overall, the routes lead 'downhill in the energy landscape' towards an energy minimum [7]. This limits the number of conformations that can be sampled and solves Levinthal's paradox.

## Folding of nascent proteins

Protein folding of a newly synthesized protein can start as soon as the N-terminus of the nascent peptide emerges from the ribosome channel. A protein may be able to reach its native conformation without assistance, but this is unlikely in the crowded environment of the cell where the risk of aggregation is high. Therefore, a multitude of folding factors is present. These chaperones and folding enzymes can catalyze slow folding steps, prevent unproductive interactions with other proteins or prevent proteins from getting trapped in off-pathway intermediates. Chaperones and folding enzymes smooth the energy landscape so that nascent polypeptides are more likely to reach their native conformation. The set of chaperones with which a nascent peptide interacts depends on the fate of the protein. A cytoplasmic protein first interacts with ribosome-associated chaperones [heat shock cognate 70 (Hsc70) and heat shock protein 40 (Hsp40) in eukaryotic cells; trigger factor in prokaryotes], and then is handed over to the cytoplasmic folding machinery (see review in [8,9]). Proteins destined for the mammalian endoplasmic reticulum (ER) are co-translationally translocated and folded by the ER chaperoning machinery. In yeast, some proteins are translocated post-translationally, after interaction with cytosolic chaperones. A general danger during protein folding, whether in the cytosol, ER or mitochondria, is the exposure of hydrophobic residues, which form undesirable interactions within or between different polypeptide chains, leading to misfolding and often aggregation. Hsp70(-like) chaperones present in all cellular compartments help to prevent this, keeping newly synthesized proteins in a folding-competent state [10]. Protein folding in the ER involves two additional features which distinguish the process from folding in the cytosol: disulfide bonds can be introduced, which covalently link two cysteine residues, and *N*-linked glycans can be attached to the folding proteins. Specialized chaperones and folding enzymes are involved in these processes. Therefore, ER-resident chaperones and folding enzymes can be divided roughly into two categories: those exerting functions exclusive for folding in the ER, and those with homology to cytosolic and mitochondrial folding factors. In the discussion below, we focus on the ER-specific folding enzymes and only briefly summarize what is known about the ER homologs of the cytoplasmic chaperones. Protein folding in the cytoplasm has been reviewed recently [7–9,11].

## The ER is a specialized folding factory

The N-terminus of a co-translationally translocated protein often functions as a signal peptide [12], which is recognized by a signal recognition particle (SRP). Binding of SRP will stall translation temporarily and target the ribosome to a translocon pore in the ER membrane [13]. The mRNA itself may direct the translating ribosome to the ER membrane as well [14]. When translation is resumed and SRP is released, the nascent chain enters the ER, where it is welcomed by a well-equipped team of proteins that assist folding.

ER-resident chaperones and folding enzymes greatly outnumber the client proteins that need to be folded, reaching concentrations close to the millimolar range [15,16]. Proteins that have not folded correctly interact with ER-resident folding factors until they reach their native conformation. If the folding process fails, they eventually are released from the folding factors to be retrotranslocated to the cytosol, where they are degraded (see below). When a client protein has folded correctly, it is transported out of the ER towards its final destination. In this way, a high folding factor to client ratio is maintained. Figure 1 shows the various processes and chaperone machineries that are described below.

The folding machinery of the ER assists the folding of a wide range of clients. One-third of all proteins expressed in *Saccharomyces cerevisiae* fold in the ER and, for humans, this percentage may be even higher [17,18]. The diverse repertoire of ER-resident folding factors reflects this diversity of clients: multiple members have been identified for several families of chaperones and folding enzymes (Table 1). In addition, the

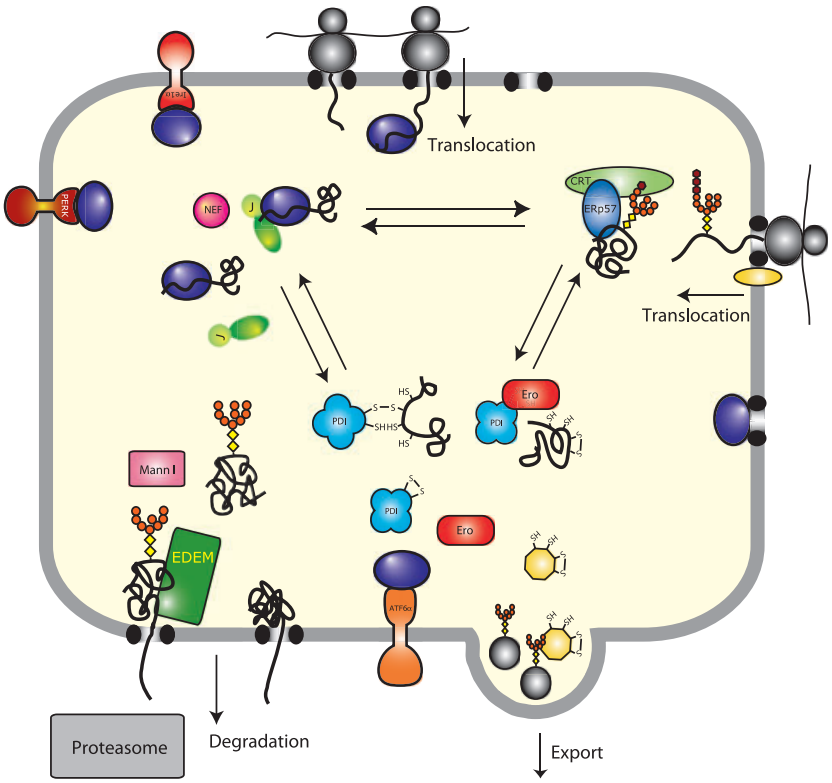


**Fig. 1.** Protein folding supported by the ER. A newly synthesized protein enters the ER through the translocon, starts to fold and may become glycosylated. It immediately associates with one of the folding factor machineries, depending on its characteristics, which include hydrophobicity, free cysteines and glycans. A folding protein may be handed over from one chaperone system to the next, using them in sequence, or may use only a single chaperone. When the preferential chaperone is not available, another one may take over. If released from all chaperone systems and hence considered to be correctly folded, the protein is ready to leave the ER. If misfolded, it will be handed over to the degradation machinery. If misfolded proteins accumulate, stress sensors are activated.

number of known private chaperones is increasing. Private chaperones have been found for various proteins in the ER. Well-known examples are the chaperones RAP and Boca/Mesd for the low-density lipoprotein (LDL) receptor family of proteins [19–22].

The ER has a high folding capacity. Specialized secretory cells, such as antibody-producing plasma cells, are capable of folding and assembling antibody molecules at high rates. CH12-LBK cells can secrete 3000 IgM molecules per cell per second [23]. Both the folding and assembly of antibodies take place in the ER (see below). Other heavily secreting cells can be found in the liver, pancreas and brain.

## Members of the ER folding crew

### Hsp70(-like) proteins and their cofactors

Hsp70 chaperones present in the cytosol, mitochondria, nucleus, chloroplast and ER aid folding by shielding exposed hydrophobic stretches so that proteins do not aggregate, keeping newly synthesized proteins in a folding-competent state [10]. BiP, the ER-resident lumenal Hsp70 [24], is an abundant chaperone that binds unfolded nascent polypeptides [25]. Peptide binding studies have confirmed that BiP has a preference for peptides with aliphatic residues, which usually are found on the inside of folded proteins [26,27]. Like other Hsp70s, BiP has an N-terminal ATPase domain and a C-terminal substrate binding domain. These domains communicate, as cycles of ATP hydrolysis and ADP to ATP exchange are coupled to cycles of substrate binding and release [28] (Fig. 2). The interdomain linker is crucial in communicating substrate and nucleotide binding from one domain to the other, which is accompanied by major conformational changes in both domains [29–32].

During its activities, BiP interacts with cofactors, many of which belong to the Hsp40 family. Five members of this family, named ERdj1–5, have been identified as ER-resident proteins [33–37]. ERdj1–5 all contain a J-domain, which can stimulate ATPase activity of BiP [29,38,39], as well as broaden the range of peptides that can bind to BiP [40]. The different topologies of the ERdjs (lumenal or transmembrane with a cytosolic domain) and their other interaction partners may fine tune BiP activity. Phosphorylation of the cytosolic C-terminus of ERdj2/Sec63p, for instance, can regulate the availability of BiP for newly translocated proteins. The recognition of yeast proteins that are translocated post-translationally is mediated by Sec62p, which forms a complex that includes Sec63p. The stability of this complex is mediated by the phosphorylated C-terminal domain of Sec63p [41].

**Table 1.** ER resident folding factors. Names and accession numbers of ER resident folding factors are listed per family. Accession numbers refer to human SWISS-PROT or TrEMBL accession numbers. Substrate specific chaperones, proteins only involved in (retro)translocation and the OST subunits are not included in this list. Adapted from [124,279].

| Function | Family | Mammalian name | Accession number | Yeast name |
|---|---|---|---|---|
| Oxidoreductases | Thioredoxin | PDI | P07237 | PDIp |
| | | | | Eug1p |
| | | | | Mpd1p |
| | | | | Mpd2p |
| | | | | Eps1p |
| | | PDIR | Q14554 | |
| | | PDIP | Q13087 | |
| | | PDILT | Q8IVQ5 | |
| | | P5 | Q15084 | |
| | | ERp18 | O95881 | |
| | | ERp27 | Q96DN0 | |
| | | ERp29 | P30040 | |
| | | ERp44 | Q9BS26 | |
| | | ERp46 | Q8NBS9 | |
| | | ERp57 | P30101 | |
| | | ERp72 | P13667 | |
| | | ERdj5[a] | Q8IXB1 | |
| | | TMX | Q9H3N1 | |
| | | TMX2 | Q9Y320 | |
| | | TMX3 | Q96JJ7 | |
| | | TMX4 | Q9H1E5 | |
| | PDI/Erv | QSOX1 | O00391 | |
| | | QSOX2 | Q6ZRP7 | |
| | Erv | | | Erv2p |
| | Ero | Ero1α | Q96HE7 | Ero1p |
| | | Ero1β | Q86YB8 | |
| Glycosylation | Glycan | Glucosidase I | Q13724 | Gls1p |
| | modification | Glucosidase II α subunit | Q14697 | Gls2p |
| | | Glucosidase II β subunit | P14314 | |
| | | UGGT | Q9NYU2 | |
| | | α Mannosidase-I | Q9UKM7 | Mns1p |
| | Lectin | Calnexin | P27824 | Cne1p |
| | | Calreticulin | P27797 | |
| | | Calreticulin 2 | Q96L12 | |
| | | Calmegin | O14967 | |
| | | EDEM1 | Q92611 | Htm1p |
| | | EDEM2 | Q9BV94 | |
| | | EDEM3 | Q9BZQ6 | |
| Chaperones | Hsp90 | GRP94 | P14625 | |
| | Hsp70 | BiP[a] | P11021 | Kar2p[a] |
| | Hsp110 | GRP170[a] | Q9Y4L1 | Lhs1p[a] |
| | Co-chaperones | ERdj1 | Q96KC8 | |
| | | ERdj2 | Q9UGP8 | Sec63p |
| | | ERdj3 | Q9UBS4 | |
| | | ERdj4 | Q9UBS3 | |
| | | ERdj5[a] | Q8IXB1 | |
| | | BiP[a] | P11021 | Kar2p[a] |
| | | GRP170[a] | Q9Y4L1 | Lhs1p[a] |
| | | BAP/Sil1 | Q9H173 | Sil1p |
| Peptidyl-Prolyl cis-trans | CyP | CypB | P23284 | Cpr5p |
| isomerases | FKBP | FKBP2 | P26885 | Fkb2p |
| | | FKBP7 | Q9Y680 | |
| | | FKBP9 | O95302 | |
| | | FKBP10 | Q96AY3 | |
| | | FKBP11 | Q9NYL4 | |
| | | FKBP14 | Q9NWM8 | |

[a] Placed in two subclasses.

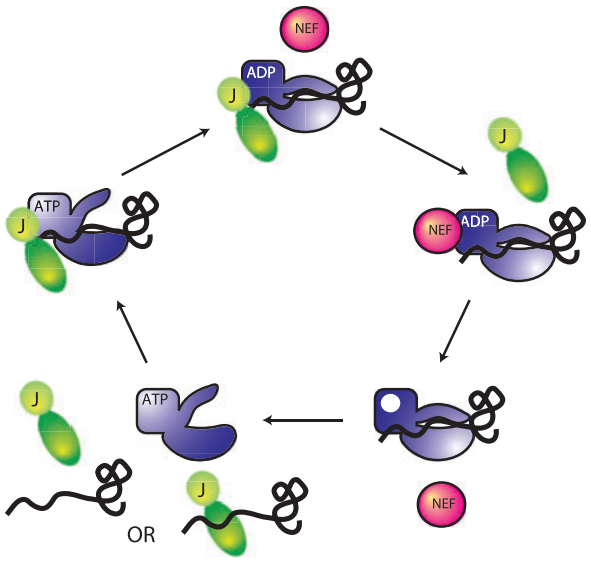


**Fig. 2.** The Hsp70 chaperone BiP ATPase cycle. The cycle starts by the binding of substrate, which may be presented by one of the five J proteins in the ER. J then stimulates BiP's ATPase activity and bound ATP is hydrolyzed, leading to a conformational change in BiP, which closes the lid domain and drastically decreases the on and off rates of substrate from BiP. One of the two nucleotide exchange factors then mediates the release of ADP, allowing the binding of ATP, which opens the lid to release the substrate for another round.

Recently, the importance of ERdj2 in humans has been illustrated by the finding that mutations in ERdj2 cause polycystic liver disease, in which fluid-filled biliary epithelial cysts are formed in the liver [42,43].

Two nucleotide exchange factors have been identified for BiP: BiP-associated protein (BAP) [44] and glucose-regulated protein 170 (GRP170) [45]. GRP170 has a dual role in the ER, as it is an Hsp110 homolog and therefore also a member of the Hsp70 family, and acts as a chaperone for ER clients [46]. In yeast, the ATPase activity of GRP170 has been shown to be stimulated by BiP [47]. The two proteins thus cooperate in assisting protein folding. BiP and GRP170 probably differ in their substrate specificity, however, as shown for the yeast homolog of GRP170, Lhs1p. Lhs1p is not necessary for *de novo* folding of several substrates, but is required for refolding of these substrates after heat shock-induced misfolding [48]. BiP (and its yeast ortholog Kar2p), by contrast, interacts with newly synthesized proteins [49,50].

## GRP94, an ER-resident Hsp90 homolog

GRP94, also known as endoplasmin, gp96 or CaBP4, is the ER-resident Hsp90. It is one of the most abundant ER-resident chaperones [51] and, as with other lumenal proteins, GRP94 has a high calcium binding capacity, making it an important calcium buffer [52]. Hsp90 and GRP94 share the same domain organization (an N-terminal domain with an ATP binding pocket [53], a middle domain and a C-terminal domain), which is essential for dimerization [54]. Elucidation of the structure of GRP94 in different nucleotide-bound states as well as investigations into the ATPase cycle of GRP94 show differences from Hsp90, however. Although the N-terminal domain binds ATP, the structural maturation of the substrate has been proposed to serve as the signal for dissociation of the complex rather than ATP binding or hydrolysis, which was initially thought not to take place in GRP94 [55,56]. Dollins *et al.* [57] and Frey *et al.* [58] have shown recently, however, that the ATPase activity of GRP94 is comparable with that of yeast Hsp90, although the conformational changes undergone by Hsp90 during the cycle are not seen for GRP94. GRP94 can change between an open and a closed conformation, but both conformations exist in the ATP- and ADP-bound states [57]. The agent that drives the chaperoning cycle of GRP94 remains to be elucidated; it may involve yet unidentified cofactors or the client proteins themselves. Two recent studies of Hsp90 homologs in solution [59,60] have provided evidence that the Hsp90s are highly dynamic structures able to adopt conformations that are not always seen in the crystal structures. It is probable that, in the near future, more information about the dynamics of the different Hsp90s in the apo-, GDP- and GTP-bound forms will become available, leading to the determination of the chaperoning mechanism.

GRP94 has peptide binding capacity, but seems to recognize a more specific subset of clients than does BiP [61]. GRP94 interacts with major histocompatibility complex (MHC) class II, but not the structurally related MHC class I chains [62]. It also interacts with late, but not early, folding intermediates of the Ig light chain, which are handed over from BiP [63]. It has also been shown to interact with a variety of receptors, including several Toll-like receptors, insulin-like growth factor receptors and integrins [64]. This substrate specificity suggests that GRP94 binding depends on more than just the exposure of hydrophobic stretches.

## Peptide bond isomerases

Peptide bonds are synthesized in the *trans* configuration on the ribosome [65], and most peptide bonds in folded proteins are in this conformation because it is

lower in energy than the corresponding *cis* configuration [66]. This is different for the peptide bond between an amino acid and a proline (X–Pro), however, as the *cis* and *trans* configurations are nearly equal in energy [67]. Depending on the side-chain, 6–38% of the X–Pro peptide bonds are in the *cis* configuration in folded proteins [68].

Spontaneous isomerization is a very slow process, but prolyl-peptidyl isomerases (PPIases) catalyze the reaction [69]. PPIases are classified into three families based on their binding to specific immunosuppressive drugs. Members of two of these classes have been identified in the ER: cyclophilin B (CypB) of the cyclophilin family and six members of the FK506 binding proteins (Table 1). CypB inhibition has been shown to retard the triple helix formation of collagen [70] and the maturation of transferrin [71], and CypB binds and affects HIV Gag and the HIV capsid protein p24 [72,73]. Although complexes between PPIases and other folding factors have been described [74–76], little is known about the function of the different PPIases in the ER.

Despite the higher energy of the *cis* configuration of 'normal' peptide bonds, they do occur in several proteins and the transition from *trans* to *cis* can be a rate-limiting step in folding [77]. The bacterial Hsp70 homolog, DnaK, was the first protein identified to catalyze this reaction, and mammalian homologs followed [78]. The function of Hsp70s thus seems to be broader than anticipated previously.

## Protein disulfide isomerase (PDI) and its family members

Most proteins that fold in the ER contain disulfide bonds. The oxidation of cysteine residues into disulfide bonds occurs during the folding process (reviewed by Tu and Weissman [79]), and is essential for proteins to reach their native structure [80]. Moreover, the prevention of oxidation eventually leads to apoptosis [81]. Why are disulfide bonds so important? During folding, they may restrict the flexibility of the polypeptide, giving directionality to the folding process, and may provide additional stability to the folded protein. Once folded proteins have left the ER, folding assistance is no longer available to reverse unfolding events, unlike in the cytosol or mitochondria.

PDI is the prototype of the ER oxidoreductase family, which introduces and reduces disulfide bonds in client proteins [82] (Fig. 3). PDI has four thioredoxin domains and a C-terminal acidic domain which binds calcium [83]. The thioredoxin domains are labeled '**a**', '**b**', '**b′**' and '**a′**' in order of appearance. The two catalytic **a** domains have a conserved CXXC motif, which is the redox-active site. When PDI functions as an oxidase, the two cysteine residues form an unstable disulfide bond and, via a mixed disulfide, this bond is transferred to the client protein [84]. Apart from oxidizing substrates, PDI also has the ability to reduce and isomerize disulfide bonds, the latter by direct rearrangement of intramolecular disulfide bonds [85] or by cycles of substrate reduction and subsequent oxidation [86]. The active sites of most PDI family members consist of a CGHC motif. The central and immediately surrounding residues are important in determining the p$K_a$ values of the active site cysteines, and therefore the preference for oxidation or reduction of disulfide bonds [87,88].

The crystal structure of yeast PDI (PDIp) revealed that the four thioredoxin domains are arranged in the shape of a 'twisted U', with the two active sites facing each other, suggesting cooperativity between the active

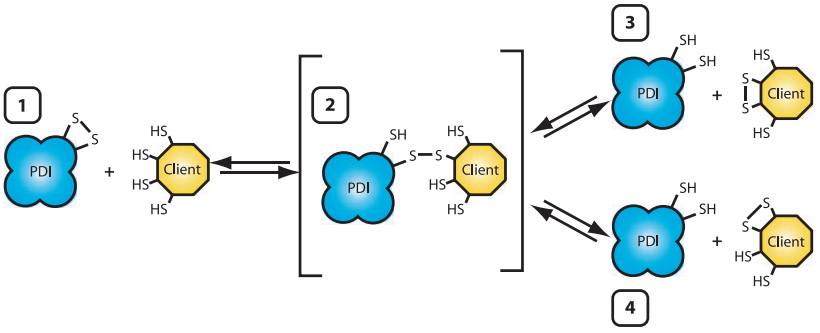


**Fig. 3.** PDI catalyzes disulfide bond formation in the ER. When the CXXC motif of PDI's active site is oxidized (1), PDI can catalyze the formation of disulfide bonds in a client protein via the formation of a mixed disulfide bond (2). When reduced (3 and 4), PDI can function as a reductase or isomerase. The isomerization reaction may proceed directly (3 → 2 → 4), or in two steps by reduction of the disulfide bond by one PDI, followed by the oxidation of different cysteines by a second PDI molecule (3 → 2 → 1 → 2 → 4). The other 24 ER-resident oxidoreductases may also catalyze at least one of these reactions.

sites [89]. Several hydrophobic patches were identified on the surface of PDIp, forming a continuous hydrophobic surface which may be crucial for interaction with partly folded substrates [89]. The **b′** domain contains the principal peptide binding site [90], and PDI has chaperone activity as well as oxidoreductase activity [91]. Interaction with unfolded substrates does not depend on PDI's oxidoreductase activity [92], as PDI can also act as a chaperone for proteins without cysteines [93]. Therefore, chaperone activity and oxidoreductase activity are not necessarily coupled.

PDI is not the only oxidoreductase in the ER. In humans, 19 other ER-resident proteins with at least one thioredoxin-like domain have been identified, and the list is still growing (Table 1) [94]. The family members differ from PDI in domain organization, tissue specificity and/or sequence of the active site. A few examples are given below.

ERp57 is an extensively studied family member. Like PDI, it has an '**a**, **b**, **b′**, **a′**' domain organization. By contrast with PDI, ERp57 closely associates with the lectins calnexin and calreticulin (see below and Fig. 4), and hence is specialized in glycoprotein folding [95,96]. By contrast with PDI, the **b′** domain of ERp57 is not used for substrate binding and chaperone activity, but forms the interaction site with the lectin [97]. Therefore, substrate specificity is probably defined by the lectin, which acts as an adaptor molecule [97]. Jessop *et al.* [98] recently identified endogenous substrates of ERp57 by trapping them as mixed disulfides with the oxidoreductase. Most substrates were found to be heavily glycosylated disulfide bond-containing proteins with common structural domains [98].

Both PDIp and PDILT are expressed in a tissue-specific manner. PDIp is a close homolog of PDI in terms of domain organization and sequence of the active site, but expression is restricted to the pancreas [99]. PDILT is a testis-specific protein with a nonclassical SXXC active site [100].

ERdj5 contains both thioredoxin domains and a J-domain [35]. The four **a** domains have CSHC, CPPC, CHPC and CGPC active sites. The CXPC motifs are similar to those of thioredoxins, proteins involved in the reduction of disulfide bonds in the cytosol and mitochondria [101]. Via its J-domain, ERdj5 interacts with BiP [35], which puts ERdj5 in place to coordinate disulfide bond formation/isomerization, chaperoning and perhaps even translocation, somewhat similar to the coordinated activities of calnexin or calreticulin and ERp57 [96].

Related but different from the thiol-oxidoreductases are two selenocysteine-containing proteins in the ER. Selenocysteines are rare amino acids that resemble cysteines, but a selenium atom replaces the sulfur atom. Like two cysteines, two selenocysteines can form a covalent bond between two residues. The ER-resident

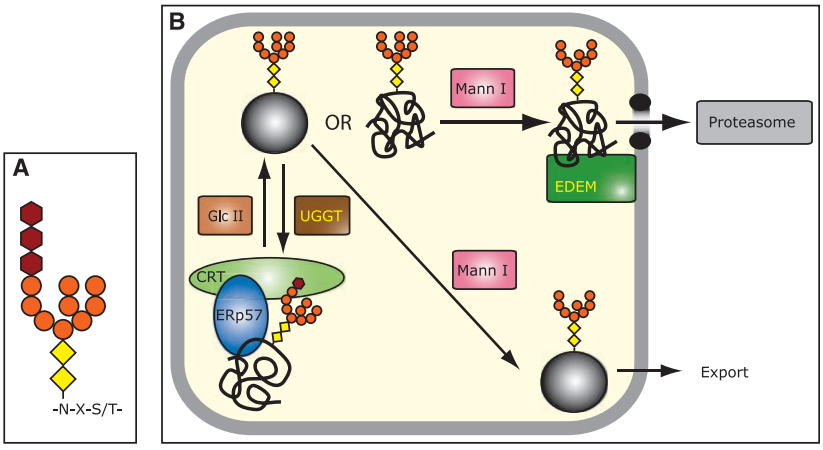


**Fig. 4.** Glycan-mediated chaperoning in the ER. (A) Structure of the preformed glycan unit ($GlcNAc_2$-$Man_9$-$Glc_3$) that is attached to the consensus glycosylation site in the polypeptide. (B) Glycoproteins enter the calnexin/calreticulin pathway after trimming of two glucose moieties by glucosidases I and II. Trimming of the third glucose by glucosidase II releases the glycoprotein from the calreticulin/ERp57 or calnexin/ERp57 (not shown) complex. Reglucosylation by UGGT enables another round of interaction with calnexin or calreticulin. α-Mannosidase I can cleave mannose residues from the glycan structure to form the Man8B isomer. If the protein is correctly folded, it can leave the ER. If the protein is terminally misfolded, further mannose trimming by α-mannosidase I enables the interaction with proteins of the EDEM subfamily, after which client proteins are retrotranslocated and degraded by the cytoplasmic proteasome complex. Correctly folded protein is indicated by a filled symbol; protein in the non-native state is indicated by a black 'squiggly' line. CRT, calreticulin; Glc II, glucosidase II; Mann I, α-mannosidase I.

selenocysteine-containing proteins Sep15 and SelM have NMR structures reminiscent of a thioredoxin domain with CXXC-like active sites [102]. Sep15 interacts with UDP-glucose:glycoprotein glucosyltransferase (UGGT; see Lectin chaperones) [103]. These proteins may be novel members of the ER folding factory whose role has not received much attention to date.

The multitude of PDI family members reflects both the importance and difficulty of introducing correct disulfide bonds into client proteins. Reaching the correct oxidized structure often requires extensive shuffling of non-native disulfide bonds [104,105]. All of the different family members may have their own expertise in assisting either specific clients or different stages in the folding process. Indeed, Winther and coworkers [106] have shown that, in *S. cerevisiae*, the five PDI homologs are not functionally interchangeable. In mammalian cells, the differences between PDI family members are illustrated by the opposing roles played by PDI and ERp72 in retrotranslocation. Forster *et al.* [107] found that PDI facilitated retrotranslocation of cholera toxin and misfolded protein substrates, whereas ERp72 mediated their retention in the ER.

## Endoplasmic reticulum oxidoreductin 1 (Ero1) proteins

The active site of PDI needs to be recharged after oxidizing a client protein. A long-standing debate on how this is accomplished was terminated by the identification of Ero1p in a screen for yeast mutants defective in disulfide bond formation [108,109]. This elucidated a pathway whereby electrons can flow from PDIp via Ero1p and FAD to molecular oxygen [110]. Ero1p directly oxidizes the CXXC motif of PDIp [84].

In mammalian cells, there are two Ero1p homologs: Ero1α and Ero1β [111,112]. The two homologs show different tissue specificities and regulation, with Ero1β upregulated by the unfolded protein response (UPR, see below) [112,113] and Ero1α only by hypoxia [114]. Mammalian Ero1α or Ero1β and PDI interact directly, as do their yeast homologs [115]. In addition, mixed disulfide bonds were found for Ero1α and Ero1β with ERp44, another PDI family member [116,117]. ERp44 has a nonclassical CXXS active site and therefore cannot act as an oxidase on its own. It does, however, retain Ero1α and Ero1β in the ER, as these proteins do not have known retention signals [116,118].

The characteristic elements of both yeast and mammalian Ero1 proteins are the bound flavin cofactor FAD, a catalytic CXXCXXC motif and a thioredoxin-like dicysteine motif. The structure of yeast Ero1p and follow-up studies with Ero1p mutants have provided insight into the mechanism through which Ero1p can shuttle electrons from PDI to molecular oxygen [119]. The dicysteine motif, present on a flexible segment of the polypeptide, interacts with PDI to accept its electrons [120]. These are then shuttled to the catalytic cysteines in the CXXCXXC motif by inward movement of the flexible segment to bring the cysteines in close proximity [119]. This flexibility, and hence electron shuttling and Ero1p activity, is hampered by two structural disulfide bonds that first need to be reduced for Ero1p to become active, an elegant regulatory mechanism that prevents hyperoxidation of the ER by Ero1p [121]. Finally, the bound FAD cofactor can shuttle the electrons to molecular oxygen or other electron acceptors [122]. Although their sequences are not similar, Ero1 appears to share structurally conserved catalytic domains with DsbB, a protein found in the periplasmic membrane of Gram-negative bacteria [123], the functional equivalent of the eukaryotic ER. Mechanisms of disulfide bond formation and isomerization, as well as the exact transport routes for electrons, have been characterized extensively in bacteria (see [124] and references therein).

## Lectin chaperones

*N*-Linked glycosylation of asparagine residues in an N–X–S/T motif is an ER-specific protein modification. Preformed oligosaccharide units, $GlcNAc_2$-$Man_9$-$Glc_3$ (Fig. 4A), are transferred *en bloc* by the oligosaccharyl transferase complex as soon as the nascent chain enters the ER lumen [125]. Indeed, when folding proceeds, glycan acceptor sites can become buried and remain unmodified, showing that folding and glycosylation compete *in vivo* [126]. The function of *N*-glycans is multifold: during folding they direct the association with lectin chaperones, increase the solubility of the polypeptide and may influence its local conformation. Once the protein is folded, glycans participate in many key biological processes, such as self/non-self recognition in immunity, signal transduction and cell adhesion [127].

Glucose trimming by glucosidases I and II produces a monoglucosylated species that can bind to the lectin chaperones calnexin and calreticulin [128–130] (Fig. 4B). The two proteins are highly homologous, apart from the fact that calnexin is a transmembrane protein and calreticulin is soluble. Calnexin is thought to interact with glycans closer to the membrane, whereas calreticulin binds more peripheral glycans [131,132]. Although both proteins associate with both soluble and membrane proteins, they interact with a distinct set of client proteins. This may partly be the result of their different localization in the ER because,

when the transmembrane segment of calnexin was fused to calreticulin, the pattern of associating proteins shifted towards that normally seen for calnexin [133]. Despite their homology, however, the two lectins are not fully interchangeable. For example, some subunits of the T-cell receptor (TCR) interact only with calnexin [134], calnexin depletion prevents the correct maturation of influenza hemagglutinin but does not interfere with the maturation of the E1 and p62 glycoproteins of Semliki Forest virus [131], and, in the absence of functional calnexin, most substrates associate with BiP rather than with calreticulin [132].

The release of substrate requires the removal of the last glucose residue by glucosidase II. UGGT can then act as a folding sensor (Fig. 4B): it has affinity for hydrophobic clusters present in glycoproteins that are in a molten globule-like state [135]. When these are detected, UGGT reglucosylates a trimmed glycan nearby, enabling renewed calnexin/calreticulin binding [136,137]. Proteins do not cycle between UGGT and calnexin/calreticulin indefinitely, however, and those that fail to fold need to be removed from the ER.

## Quality control: transport, retention or degradation?

Most proteins that fold in the ER ultimately need to leave this compartment and travel along the secretory pathway to their final destination. As long as proteins are not correctly folded, they interact with chaperones or oxidoreductases, which prevents aggregation. When a client protein is stable without chaperone binding, it can leave the ER. Retention of folding intermediates by chaperones is commonly referred to as quality control: it ensures that only correctly folded proteins are released from the ER.

A fraction of client proteins never fold into a transport-competent state and need to be disposed of to maintain cellular homeostasis. In a process called endoplasmic reticulum-associated degradation (ERAD), proteins are retrotranslocated to the cytosol where they are degraded by the proteasome [138]. A distinction needs to be made between proteins that do and proteins that do not carry a glycan. For glycoproteins, a degradation pathway has been elucidated (Fig. 4B; reviewed by Lederkremer and Glickman [139]). Resident ER mannosidase I and possibly other mannosidases remove the outermost mannose residues in glycoproteins. Glycans that are trimmed to $GlcNAc_2Man_8$ are recognized by another group of lectins, the three endoplasmic reticulum degradation-enhancing α-mannosidase-like (EDEM) proteins [140–143], which target the attached proteins for degradation (reviewed by Olivari and Molinari [144]). Proteins to be degraded are ubiquitinated. The cell uses different ubiquitin ligase complexes to 'tag' different classes of protein (misfolded lumenal, misfolded transmembrane and proteins with misfolded cytosolic domains), suggesting that there are different ERAD pathways for different glycoproteins [145,146]. The recognition of nonglycosylated ERAD substrates has received less attention, but recently two studies have shown that, as nonglycoproteins are substrates of GRP94 or BiP, their ERAD pathways do not completely overlap with those for glycoproteins [147,148]. BiP and PDI have been shown to be involved in ERAD by targeting a β-secretase isoform for degradation [149]. How and whether BiP and PDI can discriminate between folding intermediates and folding failures is unclear, and provides interesting opportunities for further research [150].

Although changes in local structure can be sufficient to retain a protein in the ER [151], retention is not always this strict. Mutations in the ligand binding domain of the LDL receptor that cause hypercholesterolemia because of impaired LDL binding do not prevent the protein from leaving the ER and traveling to the cell surface [152]. This is just one of many examples underscoring that quality control is based on structural and not functional criteria.

## Organization of the ER-resident folding factors

### Retention of ER-resident proteins and folding intermediates

The ER accommodates a continuous flow of proteins. Newly synthesized proteins enter the ER through the translocon complex, and fully folded proteins leave the ER at exit sites, where coat protein complex II (COPII)-coated buds are loaded with cargo to mediate transport via the intermediate compartment to the Golgi apparatus [153–155]. To maintain homeostasis and prevent the escape of folding intermediates and misfolded proteins, resident ER proteins and incompletely folded client proteins need to be excluded from exit. In the case of escape of ER-resident proteins to the Golgi apparatus, these proteins are transported back to the ER.

Most lumenal ER-resident proteins contain a C-terminal retrieval signal that is recognized by the KDEL receptor localized in the Golgi apparatus, which functions in pH-dependent retrieval to the ER [156,157]. The receptor recognizes KDEL, but also variations in this motif [158]. ER-resident type I and type II transmembrane proteins contain a di-lysine or di-arginine

motif, respectively, in their cytosolic terminus. ER retrieval occurs via direct interactions of these motifs with coat protein complex I (COPI), which functions in vesicular trafficking and retrieval of proteins from the Golgi apparatus to the ER [159,160].

For a newly synthesized protein to exit the ER or, in other words, to pass the ER quality control, two conditions need to be met: (a) the protein needs to lack interactions that may retain it in the ER, and (b) the protein needs to be recognized by the export machinery of the ER. The retention of folding intermediates can be the consequence of their interaction with resident ER chaperones or folding enzymes. Exposed cysteine residues can mediate retention through mixed disulfide bonds with the ER matrix, a process called thiol-mediated retention [161]. Ero1α and Ero1β, for instance, are retained in the ER by the formation of mixed disulfide bonds with their partner proteins ERp44 and PDI [116,118].

To leave the ER, a putative cargo protein needs to enter COPII-coated vesicles, which is mediated via specific interactions of the cargo protein with the COPII Sec23/Sec24 cargo selection complex [162]. Therefore, another way to prevent transport is to mask export signals. Conversely, ER exit may be allowed by masking a retention signal, similar to the way in which 14-3-3 proteins bind to and hence regulate the cell surface expression of transmembrane proteins [163].

## Microdomains in the ER

The ER lumen contains proteins with apparently opposing functions. For example, oxidases and reductases work side by side to introduce and reduce disulfide bonds, respectively. Non-native disulfide bonds are formed during folding of the LDL receptor [104], and isomerization of these disulfide bonds starts before the completion of oxidation (J. Smit, Utrecht University, The Netherlands; personal communication). Proteins that are targeted for retrotranslocation are already reduced in the ER lumen [164,165]. Oxidation and reduction, in principle, can be performed by the same protein, as PDI has been shown to be capable of both the formation and reduction of disulfide bonds *in vitro* [86,166,167]. This implies that a single overall redox potential does not exist in the ER, but that 'microenvironments' exist that allow these opposing activities [168]. Since the discovery of the Ero1 family of proteins, the concept of 'redox milieu' in the ER has changed dramatically, as it has become clear that all redox reactions in the ER, in principle, are mediated through protein–protein interactions. Considering the high intracellular concentration of glutathione and its capacity to modify protein cysteines, small molecule thiols are unlikely to remain inert, but their precise role remains to be established.

The microenvironment in the ER may be as small as the interaction interface or as large as a lipid domain or protein complex. Subdomains in the ER have been coined from many perspectives. Calcium levels are heterogeneous throughout the ER [169], lipids may play a role, the nuclear envelope and smooth ER are well-established examples of specialized ER, and COPII-enriched exit domains are also easily recognizable subdomains. A recent electron microscopic study of the localization of EDEM1 showed that it is mainly localized in 'buds that form along cisternae of the rough ER at regions outside the transitional ER' [170]. The identification of vesicles containing EDEM and misfolded proteins suggested an exit route from the ER that is independent of COPII [170]. Similarly, a misfolded splice variant of the luteinizing hormone receptor accumulated in a 'specialized juxtanuclear subcompartment of the ER' [171]. Another previously unrecognized method of disposing of misfolded proteins occurs via selective autophagy of parts of the ER after stress (see below) [172–174]. This process may act as a backup pathway to ERAD and may help the cell to recover from severe folding stress [173].

## Chaperone complexes

In the crowded ER lumen, the resident proteins must contact each other. This does not necessarily mean that functionally relevant protein complexes are formed. However, many ER-resident proteins are organized in distinct complexes, such as the oligosaccharyl transferase complex, signal peptidase complex and the translocon complex [175–177]. Specific interactions between the translocon and the other two complexes mediate their close association, facilitating contact with emerging nascent chains [12,178]. This is efficient because both signal peptide cleavage and glycosylation are mainly co-translational processes in higher eukaryotes.

Folding enzymes and chaperones are also found in complexes, but the exact composition is not strictly defined as this varies according to the client and method used to detect the complexes [76,179,180]. Specific chaperone complexes often require cross-linking agents for their identification to stabilize the interactions within the complex during analysis. To obtain an insight into the dynamics of chaperone complexes, Snapp *et al.* [181] studied the diffusion rate of calnexin in the ER. Their results indicated that the ER lumen is a dynamic environment in which transient interactions and only relatively small complexes are formed.

Given the great variety of ER clients, and therefore their variable demands on the chaperone machinery, it is probable that the contacts made between different folding enzymes and chaperones are highly transient (as also suggested by Tatu and Helenius [180]). Some subcomplexes may be relatively stable, however, such as those of a chaperone with a cofactor, and of the translocon with associated proteins. These subcomplexes can be seen as pre-assembled folding machines, capable of assisting a specific folding step. Keeping the machines intact whilst maintaining the freedom to arrange them according to the needs of the various folding clients provides the ER with the flexibility required for a dynamic and flexible folding factory.

## The ER adapts to changing circumstances

### Fusion and fission

The morphology of the ER is continuously and rapidly changing in living cells, with tubules and sheets constantly forming and reshaping [182,183]. Cargo-loaded COPII vesicles leave the ER at exit sites, and COPI-coated vesicles fuse with the ER to deliver their retrieved load. The dynamic restructuring of the ER network is enabled by the branching of existing tubules and the fusion of tubules with each other [182]. Work by Rapoport and coworkers [184] has shown that the reticulon and deleted in polyposis 1 (DP1) protein families are involved in the shaping of ER tubules. Mechanisms to change the shape of the ER provide flexibility to alter its structural organization, which is required for adaptation to changes in cellular requirements.

### The mammalian UPR

Although the ER is not a static organelle and has a high folding capacity, several events can perturb correct functioning. The synthesis of mutant proteins that misfold beyond rescue, environmental stresses, such as heat shock or hypoxia, or a sudden increase in protein synthesis can result in overload of the ER folding capacity and the accumulation of unfolded and misfolded proteins. The ER contains sensors that detect whether the folding capacity is taxed, and, if so, adaptive pathways are activated. On the one hand, the folding capacity is increased by expansion of the compartment and upregulation of chaperones and folding enzymes; on the other, the load on the ER is decreased by attenuation of general protein synthesis and increased ERAD capacity. Collectively, these sensing and response mechanisms are termed the 'unfolded protein response (UPR)' (recently reviewed in [172,185,186]). It is important to realize that the UPR prevents stress. A cell that shows a stress response is a healthy cell without stress, because it can cope with it. When the ER stress persists, however, the UPR causes cell cycle arrest and the release of $Ca^{2+}$ into the cytosol, which then leads to apoptosis [187,188]. Interaction of one of the folding capacity sensors in the ER, inositol requiring protein 1 (Ire1), with BAX and BAK, two proapoptotic proteins, provides a physical link between UPR and apoptosis [189]. To study UPR, strong intervention with protein folding is normally used, such as the treatment of cells with dithiothreitol to prevent oxidative folding, or blocking glycosylation with tunicamycin. In the *in vivo* situation, perturbation of protein folding is likely to be less dramatic or sudden, which may result in specific activation and timing of the stress sensors.

Three main stress sensors reside in the mammalian ER: Ire1α (and its homolog Ire1β), activating transcription factor 6α (ATF6α) (and its homolog ATF6β) and PKR-like endoplasmic reticulum kinase (PERK) [190–194]. All three are transmembrane proteins with a cytosolic effector domain and a lumenal domain serving as the stress sensor (Fig. 5). The Ire1 pathway is conserved between yeast and mammals [195,196], but the ATF6 and PERK pathways are specific for metazoans.

BiP binds to the lumenal domain of Ire1α monomers. The accumulation of unfolded proteins may sequester BiP, thereby activating Ire1α [197]. The crystal structure of the lumenal domain of yeast Ire1p suggests that unfolded proteins themselves can directly bind and activate the protein via an MHC-like peptide binding site [198], but the structure of the lumenal domain of human Ire1α shows that its MHC-like groove may be too narrow for peptide binding [199]. On activation, Ire1α dimerizes and trans-autophosphorylates [192], which activates the endonuclease activity of the cytosolic domain and results in splicing of one specific mRNA [200]. This spliced mRNA is translated into X-box binding protein 1 (XBP1), a transcription factor that upregulates genes coding for ER-resident proteins with ER stress elements or UPR elements in their promoter regions [200], but also others, such as the exocrine-specific transcription factor Mist1 [201]. In addition, Ire1 mediates the rapid degradation of a specific subset of mRNAs, mainly encoding plasma membrane and secreted proteins [202,203]. This complements the other UPR mechanisms aimed at relieving ER stress.

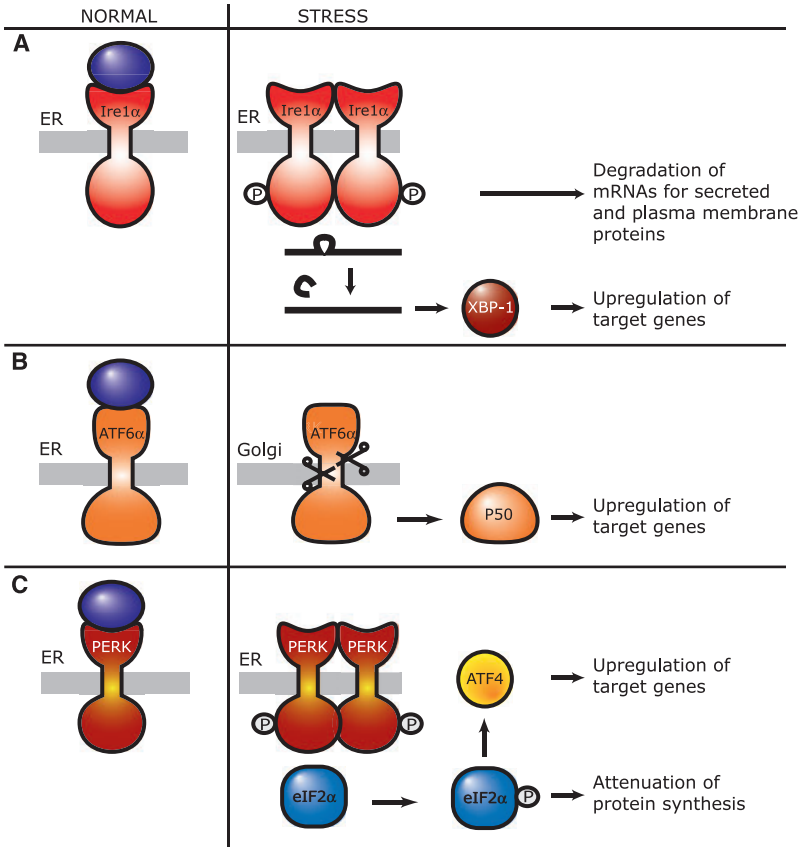


**Fig. 5.** The mammalian ER contains three main stress sensors. Ire1α (A), ATF6α (B) and PERK (C) are ER-resident transmembrane proteins with a lumenal sensing domain and a cytoplasmic effector domain. Under normal conditions, the lumenal domains interact with ER-resident proteins such as BiP. When unfolded proteins accumulate in the ER, the sensors are activated (stress), either because BiP is competed away, or because unfolded proteins may bind directly to the sensor domains. This leads to the expression of transcription factors (XBP1, ATF6, p50 and ATF4), which increases the expression of proteins encoded by UPR target genes, such as chaperones, folding enzymes and ERAD components. The burden on the ER is also alleviated by selective degradation of mRNAs encoding ER cargo (through Ire1α) and by the attenuation of general protein synthesis through the phosphorylation of eIF2α.

The second mammalian folding sensor is ATF6α. Under normal conditions, binding of ATF6α to BiP, calnexin or calreticulin mediates ER retention [204,205]. During ER stress, ATF6α travels to the Golgi apparatus where the cytosolic effector domain is cleaved off by Site 1 and Site 2 proteases [206] and acts as a transcription factor to upregulate genes with an ER stress element [206]. ATF6 contains several disulfide bonds that appear to be crucial for sensing ER stress, as these disulfide bonds are reduced on ER stress and only reduced ATF6 reaches the Golgi apparatus [207,208]. This adds a level of regulation to ATF6 activation.

PERK stimulation probably resembles Ire1α activation, because the lumenal domains of the two proteins are homologous [194]. Similar to Ire1α, PERK activation leads to trans-autophosphorylation. Phosphorylated PERK acts as a kinase that phosphorylates and inactivates eukaryotic initiation factor 2α (eIF2α) [193]. As a result, general protein synthesis is inhibited, but the translation of a subset of mRNAs is enhanced. One of these encodes the transcription factor ATF4 [209]. ATF4 promotes the transcription of a specific set of UPR target genes, distinct from those induced by XBP1 and p50 [210,211].

Although the stress sensors superficially have a similar activating mechanism, they are not always activated simultaneously [212]. For example, PERK is not activated during B-cell differentiation [213]. This creates the possibility of generating a response specific for the type and severity of stress, as the target genes of the generated transcription factors are overlapping but not identical [211].

The three arms of the UPR do not function independently of each other, however. ATF6 induces the transcription of XBP1 [200], and ATF6 and XBP1 can form active dimers regulating transcription [214]. The lumenal sensor domains may regulate the exact strength and duration of the UPR, but cytosolic proteins can also play an important role. Downregulation of Ire1 signaling in yeast, for example, is mediated by Dcr2, a phosphatase [215], and unspliced XBP1 can form a complex with the transcription factor encoded by spliced XBP1, thereby sequestering it from the nucleus and attenuating the UPR [216]. Pathways different from what is now considered to be a 'classical UPR' are also beginning to emerge, showing the integration of the above-described signaling pathways into other cellular processes. In pancreatic β cells, Ire1 can be phosphorylated and upregulates target genes, such

as *WFS1*, without XBP1 splicing [217]. During ER stress, newly synthesized proteins are degraded in a signal peptide-dependent manner by co-translational retrotranslocation and subsequent proteasomal degradation, which complements the translational inhibition by phosphorylated eIF2α [218]. One of the outcomes of UPR is increased proteasomal activation, but this is carefully timed, as during translational arrest degradation is blocked. This presumably prevents the depletion of short-lived essential proteins [219]. The identification of ATF6-like proteins and the many further examples of feedback loops and integration of multiple signaling pathways (as reviewed in [220,221]) show the sophisticated ways in which cells maintain homeostasis or adapt to changing circumstances.

The dynamic nature of complexes in the ER, the continuous dynamic restructuring of the organelle as a whole, and the presence of sensors that detect whether the level of chaperones and folding enzymes is sufficient ensure that the ER can function as a flexible protein-folding factory. This folding factory can handle the production of complicated substrates and can generate enormous output.

## Finishing folding: assembly and oligomerization

A chaperone was defined originally as a protein that is required for, or at least aids, the assembly of other proteins, but is not part of the final assembly. Later, the focus of chaperone research shifted to the role in the folding of single protein chains and in protecting the cell from adverse effects of irreversibly misfolded proteins. Yet, for many proteins, the folding process is not finished when a stable fold of the peptide chain has been attained. Proteins need to be assembled into small or large oligomers or large protein complexes. Oligomerization requires that the individual subunits find each other in the crowd of other proteins. When homotypic complexes are formed, the search for a partner is relatively simple: it can be the next protein synthesized on the same polyribosome [222]. Hetero-oligomers, or heteromers, can be formed in two ways: either by subunit exchange between homotypic complexes or by association of single subunits. Homotypic complexes may be sufficiently stable to travel unescorted, but single subunits will need to be accompanied whilst searching for their partner. Protein–protein interfaces are often hydrophobic and these hydrophobic patches need to be shielded from aberrant interaction. Single subunits may be unstable or incompletely folded and may obtain their final fold only when complexed with their partner. Oligomerization, in essence, is an extension of protein folding: the non-native protein is held by chaperones until the partner is found, at which time the protein is released into the arms of its partner. However, in at least some cases, the 'general' chaperones do not suffice for oligomerization and a chaperone dedicated to a particular subunit is required (see below for some examples). It may well be that the general folding chaperones only recognize a partially folded polypeptide, not a correctly folded subunit. In addition, the chaperone needs to hold on to the subunit until its partner is found, which is at odds with the on–off cycle of chaperone-mediated protein folding.

One of the intriguing questions in heteromer formation is how a rare protein finds its partner. In the ER, all folding membrane proteins are limited in space – the membrane – and can only diffuse laterally. One can envisage that microdomains in the membranes could serve as a trap for protein subunits and thus increase the chance of meeting a partner. What about the cytoplasm, however? Can proteins diffuse freely through this three-dimensional space or are they spatially constrained to a particular domain of the cytoplasm? What is the half-life of a lone subunit? It has been suggested that 30% of newly synthesized proteins are rapidly broken down again [223,224] (but see also [225]). Are the bulk of these proteins perhaps orphan subunits?

By contrast with the wealth of knowledge on the mechanisms of action and function of cytosolic chaperones, in general little is known about the (folding) pathways leading to a specific multimeric complex. This is different for the ER, where the detailed role of chaperones during the folding and assembly of a number of heteromeric complexes has been outlined. Below, some examples are provided of oligomer assembly in the ER and in the cytosol to illustrate the different possible pathways and proteins involved. An additional complexity of protein folding and assembly is the assembly of oligomers into even larger complexes. This process may also require special chaperones, which stabilize the intermediates, as has been found, for example, for chromatin and proteasome assembly (for a review, see Ellis [225a]).

## Oligomer assembly in the ER

### A 'simple' case: homodimer formation of thyroglobulin in the ER

Thyroglobulin (Tg) is a complex client of the ER folding factory, although it is exported from the ER as a homodimer. It is a large glycoprotein containing up to

60 disulfide bonds and 10–15 *N*-linked glycans. Tg is exported from the ER as a homodimer of 660 kDa and is secreted into the thyroid follicle, a space lined by the apical side of the thyrocytes [226,227]. Here, thyroxin and 3,5,3′-triiodothyronine are produced from the prohormone Tg by iodination of specific tyrosine residues and proteolytic cleavage of Tg [228,229].

Folding of Tg can be considered a truly demanding task for chaperones and folding enzymes, as nascent Tg forms disulfide-linked complexes with a molecular weight of over 2000 kDa [230]. In approximately 15 min these complexes dissolve efficiently into monomers [227,230], which then dimerize to become export competent. A lag time of 90 min exists between the $t_{1/2}$ of dimerization and arrival in the Golgi, indicating that dimerization *per se* is not sufficient for export [227].

The folding pathway of Tg suggests a strong requirement for chaperone assistance, and many studies have identified the chaperones and folding enzymes involved. BiP associates with Tg early folding intermediates, nascent chains, interchain disulfide bond-containing complexes, noncovalent complexes and unfolded free monomers [231]. Other folding factors implicated in Tg folding are GRP170, GRP94, ERp72, ERp29, calnexin and calreticulin [179,232–234]. The strong demand on folding factors is reflected by the simultaneous binding of multiple chaperones per Tg molecule. The average ratio of BiP/Tg is almost ten molecules of BiP per Tg molecule [231], whereas calnexin and calreticulin simultaneously bind to the same Tg molecule [235].

## A complex secreted heteromer: the case of IgM

IgM, a bulky heteromer, is the first and largest antibody to be produced in an adaptive immune response. It is secreted into the blood, where it binds antigen and activates the complement system. Like other antibodies, IgM consists of two identical heavy chains (H, μ) and two identical light chains (L, either λ or κ) that form covalently linked heterotetramers, in the antibody field called 'monomers' (Fig. 6A). Unlike most other antibodies, which are secreted in the 'monomeric' form, IgM almost always is secreted as 'hexamers' in the composition $(H_2L_2)_5$ with a third polypeptide, J-chain, as the sixth subunit [236], or $(H_2L_2)_6$ (Fig. 6A) [237]. Every μ heavy chain is glycosylated on five asparagine residues, and over 100 disulfide bonds need to form per IgM oligomer. Therefore, IgM can be considered as a demanding ER client. Both folding of the subunits and assembly of IgM occur in the ER [238]. The PDI family member ERp44 and the lectin ERGIC53 together function in the transport of assembled IgM to the Golgi [239].

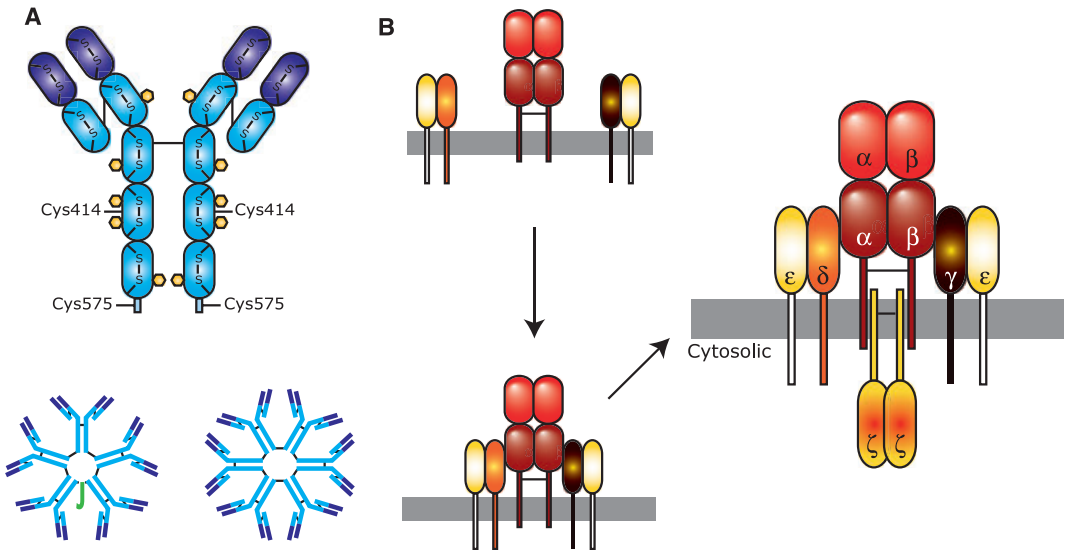


**Fig. 6.** Composition of IgM and TCR. (A) IgM 'monomers' consist of two heavy and two light chains linked by disulfide bonds. The heavy and light chains consist of several domains, each containing one disulfide bond. Constant domains are indicated in light blue and variable domains in dark blue. Conserved sites for *N*-glycosylation are indicated by hexagons. IgM is secreted as a hexamer, in which the subunits (either five 'monomers' and one J-chain, or six 'monomers') are linked by disulfide bonds between the tailpiece cysteines. (B) The TCR complex consists of a disulfide-linked dimer of the α and β chain, responsible for the recognition of the peptide presented by MHC. Subsequent signaling is mediated by the other components of the TCR complex, the δε, γε and ζζ dimers, which assemble step by step with the αβ dimer.

Heavy and light chains are composed of domains that each contain one intradomain disulfide bond. Heavy chains (μ) have one variable domain (VH) and four constant domains (CH1–4). Light chains also have one variable domain (VL), but only one constant domain (CL). Folding of the domains occurs co-translationally and proceeds from the variable to the constant domains [240], with the exception of CH1 [241]. CH1 remains unfolded and associated to BiP in the absence of CL [241–243]. Binding of BiP is constant, protecting the dimerization interface, and therefore different from the on–off cycling it displays during its 'normal' chaperone activity. CL of a folded light chain replaces BiP, and only then is the intrachain disulfide bond in CH1 formed [244]. Indeed, heavy chain is not secreted on its own, whereas light chain secretion is possible. This also suggests that heavy chain is produced in a limited amount, allowing the control of the secreted amount of the complete complex by controlling the production of one of the subunits [245].

Disulfide bonds are formed between the antibody subunits; they stabilize the $\mu_2\lambda_2$ 'monomers' and link the 'monomers' into 'hexamers'. In the 'monomer', the heavy and light chains are coupled via an interchain disulfide bond between the two constant domains, and the two heavy chains are linked through a disulfide bond between cysteines 337 in the CH2 domains. Polymerization proceeds via the formation of disulfide bonds between the tailpiece cysteines at position 575. To stabilize the polymer, additional disulfide bonds between residues 414 of the heavy chains can be formed. The tail of the heavy chain contains a highly conserved glycosylation site at position 563. The glycan attached to this site remains in a high-mannose state, indicating that it is buried in the polymer structure and therefore inaccessible to Golgi-resident, glycan-modifying enzymes [246]. The presence of this glycan is crucial for the formation of functional oligomers [247], providing an example of the importance of correct glycosylation.

Several mechanisms exist to retain assembly intermediates in the ER. The inability of the CH1 domain to fold without CL prevents the release from BiP and hence the secretion of unassembled heavy chains [248]. This may be of particular importance for antibody subtypes that do not require 'oligomerization': IgM assembly intermediates are retained through an additional retention mechanism. Cysteine 575, essential for polymerization, also mediates the retention of unpolymerized $H_2L_2$ 'monomers' by cross-linking them to proteins of the ER matrix [161,249].

Several chaperones and folding enzymes assist the folding and assembly of IgM. The role of BiP is important and well described [241–243,245], but representatives of all other known classes of ER-resident proteins (such as PDI, GRP94, GRP170, ERp72, CypB, ERdj3 and UDP-glucosyltransferase) are also involved in IgM production [63,76,167]. Whether additional B-cell-specific chaperones are involved in IgM assembly is unknown. In a proteomics study of differentiating B cells, a B-cell-specific ER-resident protein was identified, but its role in antibody production is still unclear. On expression of heavy and light chains in cells other than B cells, 'monomers' are the secreted product. Therefore, the retention of 'monomers' is specific for B cells, suggesting that cell type-specific factors must be involved.

## A membrane-bound heteromer that folds and assembles in the ER: the case of the TCR

An appropriate stoichiometry of the subunits in a hetero-oligomeric complex is important for correct functioning of the complex. The regulation of the expression of only one of the subunits provides a straightforward means of controlling the expression level of the entire complex, although it comes at the cost of investing energy and resources in producing the other subunits in excess. An example of this type of regulation is the TCR, a hetero-oligomer consisting of six different proteins. The α and β chains, both consisting of a constant and a variable domain, are linked by an intermolecular disulfide bond and are responsible for antigen recognition. This dimer interacts with the CD3 complex responsible for signal transduction, which consists of two noncovalently assembled dimers, δε and γε, and a covalently bound dimer of ζ chains [250] (Fig. 6B). Synthesis of the ζ chain is only 10% of that of the other subunits [251]. Assembly with the ζ chain confers stability to the partly assembled TCR and allows ER exit; ζ hence controls the expression of the complete receptor [251].

The assembly of the TCR occurs in a stepwise process (Fig. 6B). The signaling molecules δ, ε and γ first form δε and γε dimers, which interact with α or β chains [252]. As mentioned above, the incorporation of the $\zeta_2$ dimer is likely to be a late step in assembly and, indeed, the formation of the αβ heterodimer precedes $\zeta_2$ interaction [253]. The transmembrane regions of the TCR subunits have received considerable attention as they display characteristics common to a large number of activating receptors [254]. Both mutational and structural studies have shown that, during assembly, one basic and two acidic residues in the transmembrane regions of

the TCR subunits are required to allow interaction of the signaling dimers with the αβ heterodimer [254–257]. These same residues are sufficient to cause degradation of the subunits when assembly does not proceed [256]. Thus, the signal that allows oligomerization also provides an intrinsic quality control mechanism.

Incompletely assembled forms of the TCR are, as is the case for IgM assembly intermediates, retained in the ER, with the exception of the αβγδε form, which can travel through the Golgi, but is redirected to the lysosomes to be degraded [258]. In this way, only fully assembled TCR reaches the plasma membrane. The retention of incompletely assembled TCR in the ER presumably takes place via the interaction with chaperones, although each chain has an ER retention/retrieval signal as well, which are inactivated one by one [259]. Both calnexin and calreticulin interact with TCR subunits, although not in exactly the same manner, in that interaction with calreticulin is more transient and restricted to the α and β chains [134]. TCR-associated protein (TRAP), also called CD3ω, is found to be transiently associated with other CD3 subunits. TRAP is not present in the final complex, indicating that it may function as a private chaperone for the TCR [260].

## Oligomer assembly in the cytosol

### Folding in the arms of the subunit with a cytosolic chaperone assist: the case of caspase-activated DNase–inhibitor of caspase-activated DNase (CAD–ICAD)

Caspase-activated DNase (CAD) (also known as DNA fragmentation factor subunit β) is the enzyme responsible for cleaving DNA fragments into oligonucleosome-sized fragments during apoptosis (for a review, see [261,262]). Under normal conditions, the enzyme is complexed with its inhibitor ICAD (also known as DNA fragmentation factor subunit α), probably as a tetramer consisting of two heterodimers. ICAD is cleaved by caspase-3 and caspase-7, releasing active CAD. In apoptotic cells, CAD is found as a homo-oligomer [263]. Exogenous expression of CAD fails unless ICAD is also expressed; in the absence of ICAD, CAD is rapidly degraded [264–266]. *In vitro* refolding of CAD to an enzymatically active form requires Hsc70 and Hsp40, but also ICAD. During *in vitro* translation, ICAD as well as Hsp70 and Hsp40 associate with the nascent CAD chains, strongly suggesting that ICAD is the matrix on which CAD folds [267].

### A subunit-specific cytosolic chaperone: the case of α-globin

About 95% of the protein of a mature red blood cell is hemoglobin, a tetramer containing two α- and two β-globin subunits. Synthesis of the α- and β-globin subunits is balanced, such that there is a small excess of α-globin subunits. Neither globin subunit is very stable on its own. When the synthesis of one subunit is disturbed, as in mutations of the α- or β-globin genes, the other subunit precipitates and damages the cell. Presumably, the mechanisms that usually clear protein aggregates from the cells cannot cope which such a high level of synthesis of unstable protein. The β-globin subunit is somewhat less prone to precipitation than the α-globin subunit; it is stabilized by dimerization and tetramerization, whereas the α-globin subunit remains monomeric. The mystery of the small excess of an apparently stable variant of the α-globin monomer was solved when this monomer was found to be associated with the α-hemoglobin stabilizing protein (AHSP; [268]). AHSP binds α-globin at the same site as does β-globin, and is displaced by β-globin when the tetramer is assembled [269]. Recently, AHSP has been found to be more than just a temporary stand-in for β-globin. AHSP enhances α-globin folding during *in vitro* synthesis and refolding of denatured α-globin *in vitro* [270]. AHSP thus has all the hallmarks of an α-globin-specific chaperone.

### The subunit is the chaperone: the case of RNA polymerase (RNAP)

The eukaryotic RNAPs I, II and III are large protein complexes with an enzymatic core homologous to the prokaryotic RNAP $\alpha_2\beta\beta'\omega$ complex. The common core subunit RPB6, a homolog of the *Escherichia coli* RNAP ω subunit [271], is required for assembly of the RNAP core complex. The role of RPB6 in assembly is analogous to that of the ω subunit in the assembly of *E. coli* RNAP [272]. In *E. coli*, ω interacts specifically with the β′ subunit. *In vitro*, ω prevents the aggregation of the β′ subunit and promotes the association of β′ with the $\alpha_2\beta$ complex. The evidence that ω is involved in folding of the β′ subunits comes from experiments in which a lack of ω has been shown to be compensated by the overexpression of the cytosolic chaperone GroEL [273]. The similarity in structure and function between the *E. coli* ω protein and the eukaryotic RPB6 strongly suggests that RPB6 is a chaperone dedicated to the formation of the RNAP core complex.

### Keeping chaperoning within the family: the case of cytosolic βA4-crystallin

The β-crystallins are abundant eye lens proteins. The mammalian lens contains seven different β-crystallin proteins, encoded by a family of six genes (the seventh protein is an alternative translation initiation variant). The β-crystallins are two-domain proteins, with each domain consisting of two Greek key motifs, a very stable protein fold. They are never found as monomers, are at least dimeric, but also assemble into tetramers and octamers (for a review, see [274,275]). The β-crystallins are a group of proteins that might not require chaperoning at all: the domains fold independently; hence, the protein could fold co-translationally. They readily refold *in vitro* without assistance and form stable homodimers; heterodimers can be made *in vitro* by mixing the homodimers [276]. Therefore, it was surprising that the exogenous expression of one member of the β-crystallin family, βA4-crystallin, in a mammalian cell failed, when another member, βB2-crystallin, was abundantly expressed. The failure of expression of βA4-crystallin appeared to be the result of a folding problem, as the protein was formed but rapidly degraded. Exogenous expression of either Hsp70 or a small Hsp (Hsp27 or αB-crystallin) failed to rescue βA4-crystallin expression, but exogenous expression of βB2-crystallin did so, and led to the accumulation of βA4-crystallin as a heteromer [277]. Apparently, βB2-crystallin can capture an unstable βA4-crystallin intermediate into a stable heteromer. One of the unexpected findings was the inability of small Hsps to either stabilize βA4-crystallin or to promote βA4-/βB2-crystallin heteromer formation [278]. Two members of this class of Hsps, α- and αB-crystallin, are very abundant in the eye lens, where one of their presumed roles is to stabilize other lens proteins, such as the β-crystallins [278].

## Conclusions

Although many genomes have been sequenced and annotated and a PubMed search for the term chaperone yields more than 25 000 citations, many more new chaperones and folding enzymes are likely to be discovered in the future. Studies in complex systems still contain many unknown components, and research reports that change or challenge major concepts of how proteins fold, assemble and function appear every few years. In this review, we have discussed some well-characterized abundant or compartment-specific chaperones and folding enzymes that are part of the common general folding pathways used by many different proteins. We have also given examples of the increasing number of private chaperones, i.e. chaperones dedicated to the folding or assembly of a single protein (family). We suspect that many of the proteins that are now simply known as a 'structural' subunit of a protein assembly may well be private chaperones. The challenge will be to identify all chaperones required for the folding or assembly of a protein, and to define how these act together, simultaneously or in sequence, to produce the assembled protein. A major question is what dictates the preference of a folding protein for a particular chaperone, or vice versa.

Most mechanistic studies on chaperone action have been performed on prokaryotic proteins or their eukaryotic homologs, but folding of proteins in the intact cell has focused on mammals. Little information exists on the molecular pathways in the intact cell. In isolation, a protein can take many folding pathways; *in vivo*, this is limited to a smaller number by the cellular environment, in particular by the available chaperones. The question is whether chaperones influence a folding pathway, and whether a choice of different chaperones in different cell types or under different circumstances will change the folding pathway taken and/or the outcome of the folding process. Although the biophysical principles that govern folding *in vitro* also apply *in vivo*, and household proteins fold into a functional conformation in every cell in an organism, no evidence is yet available as to whether the fine structure of such a protein is truly identical in all cells, or whether the routes to the final structure are similar.

A nascent protein emerging from the ribosome encounters the same folding problems and follows the same basic folding rules in the cytosol and ER. The chaperones that assist the nascent chains in these two compartments are related: members of the Hsp70 family and their co-chaperones, such as the DnaJ proteins. However, once the protein is released from the ribosome, the folding pathways in the cytosol and ER may well diverge. The ER is essentially a folding factory, where folded and assembled products are sorted from misfolded proteins to be released and passed on to their final destination; misfolded proteins are retrotranslocated to the cytosol for disposal. In contrast, most of the proteins that fold in the cytosol stay in the cytosol. The cytosol does not offer a safe folding environment, but instead provides small folding chambers (e.g. Hsp60/GroEL/TriC), at least for proteins of limited size. As in the ER, cytosolic chaperones help newborn proteins; however, by contrast with the ER, cytosolic chaperones meet unfolding proteins that once were native. The same cytosolic chaperones are needed

to support proteins waiting to be activated, such as kinases and hormone receptors. How cytosolic chaperones distinguish between these different types of client is not known. Unlike in the cytosol, protein folding in the ER is dictated to a large extent by glycosylation and disulfide bond formation. Although this complicates folding studies *in vitro*, it favors the easy identification of cell biological processes in intact cells. In contrast, folding intermediates in the cytosol are much more difficult, if not impossible, to detect.

Part of this review has been devoted to oligomeric assembly, because fewer and fewer proteins are found to function in isolation. By extrapolation from the *in vitro* folding of model substrates, we presume to have some notion of how folding of single chains proceeds *in vivo*. Oligomer formation *in vitro*, however, may well not be representative of oligomer formation *in vivo*: assembly in the crowded cell amidst strangers is quite different from assembly from purified subunits in a test tube. Folding and assembly *in vivo* have been studied for so few proteins that general statements are only tentative. In the secretory pathway, oligomerization usually occurs from rather natively folded monomers in the ER, and may continue in the Golgi. In the cytosol, however, assembly often starts earlier, for a homo-oligomer perhaps already on the ribosome. During their lifetime, proteins undergo gradual conformational changes, not only forward from nascent chain to supramolecular assembly or to a misfolded monomer or aggregate, but also in the reverse direction to the unfolded state before degradation. It is clear that proteins do not travel these paths alone. The challenge for the coming years is to determine how folding proteins and their assistants influence each other's conformations and fates.

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