Chapter 3 Notes and Queries

1. Section 3.3.2, end of 2nd para: I would suggest placing a section number here, as “see below” is rather vague.

2. Section 3.4.1.3, first para: Text says: “During folding **they** are assisted by….”
To what does “they” refer here?

3. **3.5.2 (Co)-immunoprecipitation and Excessary Protocols**
I could not find this word anywhere. Should it be “accessory”?

3
Studying Protein Folding in Vivo

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3.1
Introduction

To be biologically active, proteins must fold into their three-dimensional, native structure. Christian Anfinsen and others showed decades ago that all information required for a protein to attain this three-dimensional structure resides in its primary sequence [1]. Irrespective of whether a protein folds in an intact cell, in vivo, or in a test tube, in vitro, essentially the same end product is obtained. Yet, many differences exist between the two. While in vivo folding starts co-translationally [2, 3], in vitro (re)folding starts from the complete protein molecule. The folding environment is different as well: in vivo proteins are folded in an extremely crowded environment [4–6], containing hundreds of milligrams of protein per milliliter, in contrast to in vitro folding where the protein is purified and folds in a diluted system. In vitro, folding often is inefficient under biological conditions such as physiological temperatures, with a large fraction of the protein misfolding and precipitating. In vivo, proteins are assisted during folding, and because of chaperones and folding enzymes [7–9], the folding process is more efficient and results in less aggregation than in vitro [10]. In vitro folding can take place in milliseconds, proteins can be studied directly at the molecular level, and there are no barriers such as membranes between experimenter and the folding proteins. Success rate is highest with relatively small, single-domain proteins, whereas especially mammalian proteins usually are large, consisting of more than one domain. Although in vitro folding experiments provide information on molecular details of the folding process, in vivo studies are essential for a complete view on protein folding. This chapter will discuss how protein folding in a cell can be studied and manipulated, with a slight focus on the endoplasmic reticulum.

3.2
General Features in Folding Proteins Amenable to in Vivo Study

In contrast to in vitro folding proteins, in vivo proteins can be studied only via indirect methods. Instead of direct measurements on the protein itself, the folding

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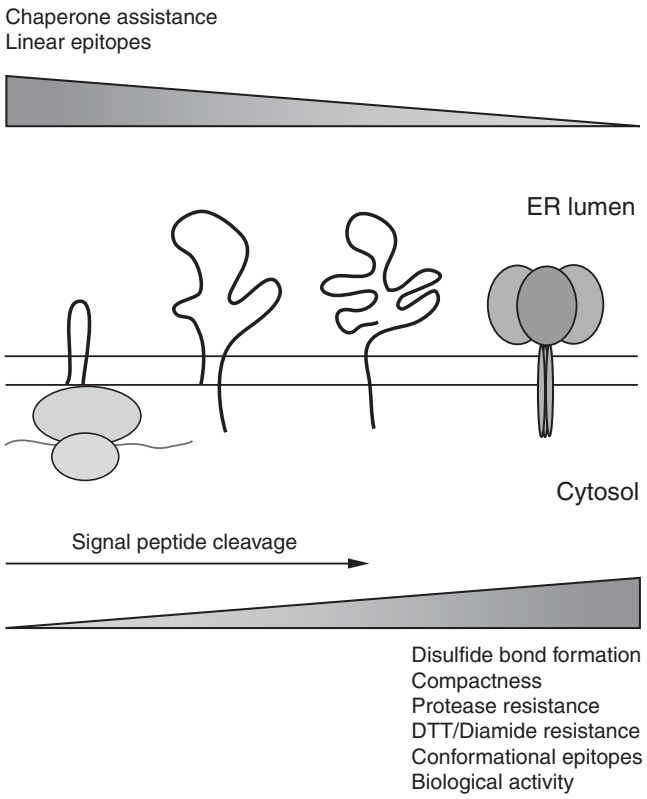

Fig. 3.1. Studying protein folding in the ER. Newly synthesized proteins that are folding in the ER lose and gain various features, which can be used to determine their folding status; examples are given in this figure. Folding starts on the nascent chain and continues after chain termination, during or after which the protein may oligomerize.

status of a protein can be followed through changes in shape, associations with other proteins, co- and posttranslational modifications, and its compartmental location (Figure 3.1). During in vivo folding, proteins are not purified but are part of the cell ''soup.'' So far the best way to follow folding of a small population of newly synthesized proteins in a cell is by labeling them with ^{35}S-labeled cysteine and/or -methionine. In this way, changes in a small, synchronized protein population can be followed with time.

A pulse-chase experiment (Figure 3.2) in intact cells is an assay in which kinetics and characteristics of sequential steps are determined in vivo. Newly synthesized proteins are labeled with radioactive amino acids for a very short pulse time, because folding starts during synthesis and one would ideally follow folding from the very first folding intermediates. Labeled proteins are ''chased'' by incubation with unlabeled amino acids. After different times of chase, cells and supernatants

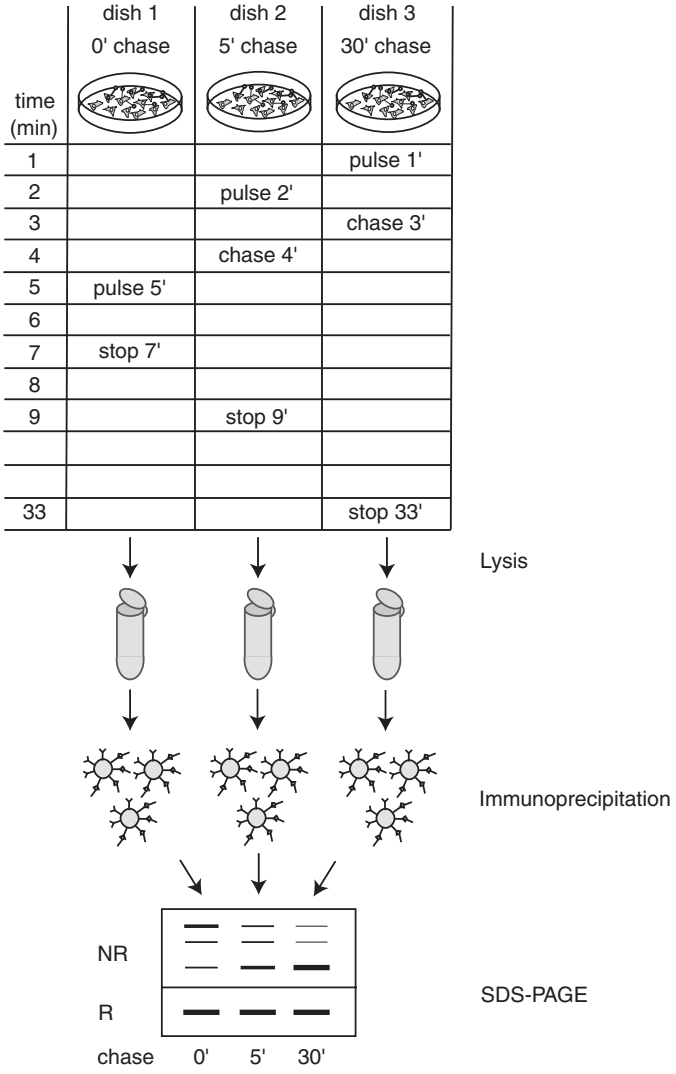

Fig. 3.2. Generic pulse-chase time schedule. To study folding and disulfide bond formation of a newly synthesized protein in the ER, adherent cells expressing the protein of interest are pulse labeled for a short period and chased with unlabeled amino acids; one dish is needed per time point. The experimental design is such that dishes can be pulse labeled and chased in parallel. After different times of chase, free cysteines are blocked with an alkylating agent and cells are lysed in a detergent-containing buffer. The protein is isolated using immunoprecipitation, and samples are analyzed using non-reducing (NR) and reducing (R) SDS-PAGE to visualize disulfide bond formation and changes in molecular weight.

can be collected and cooled on ice, and free cysteines are blocked with an alkylating agent. Cells are lysed in a detergent-containing buffer and the protein under study is isolated by immunoprecipitation. Samples can be analyzed by various types of gel electrophoresis, including native gels suitable for detection of complexes, and reducing and non-reducing SDS-PAGE to detect folding intermediates with a variable set of disulfide bonds (see the Appendix and [11]). Other changing features of folding proteins, such as their compactness or resistance to proteolytic digestion, can be determined with this pulse-chase assay as well.

Other labeling assays that can be used to study protein folding include the pulse-chase in suspension cells, microsomes, or semi-intact cell systems [12] in which mRNA is translated in vitro in the presence of a source of ER membranes (see Chapter 18) and the recently developed in vitro chase assay in which a protein is translated in vivo and chased in a detergent lysate (Maggioni et al., in press). These assays will be discussed in the Appendix.

3.2.1 Increasing Compactness

One of the general features of a protein during folding is its increasing compactness. During the folding process of a soluble protein, all hydrophobic side chains will strive to be buried inside the molecule and all hydrophilic ones to be exposed on the outside; hydrogen bonds are formed within the protein and between exposed residues and water. Electrostatic, van der Waals, and hydrophobic interactions play a major role during the folding process, and disulfide bridges will form within the protein when conformation and environmental conditions allow. The end product of the folding process will be the native conformation of the protein, which is assumed to be the most compact, energy-favorable form. To study the increase in compactness of proteins, non-denaturing gel electrophoresis can be used. The native, most compact protein will have a higher electrophoretic mobility than the more unstructured folding intermediates. The formation of oligomers, protein complexes, and transient protein-protein interactions can be detected on a native gel as well as by sucrose velocity gradient centrifugation. To monitor disulfide bond formation within or between polypeptide chains, non-reducing denaturing gel electrophoresis (SDS-PAGE) is an effective technique. In the assays described in this chapter, the folding process of a (disulfide bond containing glyco)protein can be followed kinetically.

3.2.2 Decreasing Accessibility to Different Reagents

Due to the increasing compactness of a protein on its folding pathway, it will become less accessible to various reagents including proteases. Changes in protein conformation usually result in decreased sensitivity to protease digestion, since potential cleavage sites are buried inside the protein during the folding process. To limit the appearance of protease-specific bands, a range of proteases should be

used optimally, some of which cleave frequently in a protein. Examples of the latter are proteinase K, TPCK-trypsin, and TLCK-chymotrypsin. Limited proteolysis was first used to identify domains in immunoglobulin [14] and is a very powerful tool. The target protein is exposed to a protease, and in a time course the generation of stable products and protected inaccessible domains can be monitored. To identify these domains, the digestion pattern of full-length protein can be compared to that of expressed isolated fragments of the protein, or domain-specific antibodies can be used to identify protease-resistant fragments.

Other reagents that can be used to obtain more information about the folding status of a protein include diazenedicarboxylic acid *bis*(*N*,*N*-dimethylamide) (diamide) and 4,4′-dithiodipyridine (4-DPS) [15, 16]. Diamide is an oxidant that, when added to the chase medium, will over-oxidize all compartments in the cell, resulting in oxidation of all cellular thiols, including those in proteins and glutathione. Since only proteins with free sulfhydryl groups are sensitive to diamide treatment, in the secretory pathway, only folding intermediates will oxidize, as most mature folded proteins have disulfide bonds. If treated and non-treated proteins are analyzed by non-reducing SDS-PAGE, folding intermediates and the native protein can be discriminated [17–19] (Braakman and Helenius, in preparation).

Like diamide, reducing agents such as dithiothreitol (DTT) or 2-mercaptoethanol (2ME) can be used in a pulse-chase assay to determine the folding status of a protein. The influenza virus hemagglutinin (HA) [20, 21] and other proteins with disulfide bonds [22, 23] undergo a conversion from DTT sensitivity to DTT resistance during their conformational maturation in the ER. In combination with conformation-sensitive and epitope-specific antibodies, DTT sensitivity is very useful in determining the folding hierarchy in a protein.

3.2.3
Changes in Conformation

During folding, domains are formed within a protein, coinciding with changes in conformation. Antigenic epitopes become exposed, are masked, or form because distant parts of the polypeptide chain start to interact. Conformational changes can be followed by probing the folding protein with conformation-sensitive antibodies. Contrary to general belief, completely conformation-sensitive antibodies are rare. Polyclonal as well as monoclonal antibodies generally recognize a limited conformation range of a protein, depending on how the antigen used to produce the antibody was obtained. Protein bands cut out of SDS-PA gels, when injected into rabbits, yield antibodies that work well in Western blotting (against SDS-denatured antigen) but that often fail to work in immunofluorescence, EM, or immunoprecipitation, which are conditions that allow proteins to remain more or less native. Antibodies generated against native, properly folded and assembled antigens often fail to interact with folding intermediates or SDS-denatured protein. Antibodies against peptides often fail to bind protein, because the peptide is not representative of the protein conformation or the peptide is not exposed in the

complete protein. Therefore, an antibody needs to be characterized before it becomes a powerful tool in protein-folding studies.

Another assay that can be used concerns biological function of the folding protein: this includes binding to ligands and other proteins or an increase in enzymatic activity. To test, for instance, whether the protein can bind its receptor, the receptor may be coupled to Sepharose beads, which can be used in a precipitation assay to isolate the desired protein, or, alternatively, interaction can be tested in an ELISA-like assay.

3.2.4 Assistance During Folding

All information required for a protein to attain its final three-dimensional structure resides in its primary sequence. However, complex proteins need the assistance of molecular chaperones and folding enzymes to reach their native structure efficiently without formation of (large) aggregates. Molecular chaperones interact with nonnative states of proteins; they are important during folding of newly synthesized polypeptides where they facilitate rate-limiting steps, stabilize unfolded proteins, and prevent unwanted intra- and interchain interactions, which could lead to aggregation [24–26]. Classical chaperones in the ER, such as BiP [27], recognize exposed hydrophobic patches [28, 29], while calnexin and calreticulin recognize monoglucosylated glycan chains on proteins in the ER. BiP is a member of the Hsp70 family; ATP binding and hydrolysis are crucial for the cycle of binding and release of substrate [30, 31]. The preference for binding of either BiP or calnexin/calreticulin is thought to depend on the position of the first N-linked glycan in the polypeptide chain [32]. When *N*-glycans are present in the first ~50 amino acids, a protein in general will associate with calnexin and calreticulin first, whereas proteins with *N*-glycans downstream in the sequence may bind BiP or other chaperones first. Other observations, however, suggest that calnexin/calreticulin may interact with non-glycosylated proteins as well [33, 34]. As a rule, a protein's association with molecular chaperones will decrease its folding rate but increase folding efficiency and thereby yield.

The ER in addition possesses a wealth of enzymes that assist proteins during folding. Protein disulfide isomerase (PDI) (56 kDa) [35] is a member of the thioredoxin superfamily that acts catalytically in both the formation and reduction of disulfide bonds, and hence in disulfide bond rearrangements. Another PDI family member is ERp57, which facilitates disulfide bond formation in newly synthesized proteins and works in a complex with either calnexin or calreticulin [36]. A different class of enzymes comprises the peptidyl-prolyl isomerases (PPIases) [37], which catalyze *cis*/*trans* isomerization of peptide bonds N-terminally to proline residues [38] and thereby increase folding rates [39]. The ribosome attaches amino acids in the *trans* conformation, which requires an isomerization step for all *cis* prolines in the final folded structure. PPIases belong to three structurally diverse families: the cyclophilins (inhibited by cyclosporin A [CsA]), the FK506-binding

proteins (inhibited by FK506 and rapamycin), and the parvulins. They reside in the cytoplasm and in various other organelles of the cell including the ER. In 1991 Steinmann et al. [40] and Lodish and Kong [41] showed that the folding of procollagen I and transferrin is slowed down by CsA due to inhibition of cyclophilin.

Folding proteins can be assisted by chaperones and folding enzymes from different compartments. If a protein is soluble, only the chaperones and folding enzymes that are located in the same compartment as the protein will assist folding. Membrane-bound or membrane-spanning proteins, however, will have domains in different compartments. For example, both P-glycoprotein and the cystic fibrosis transmembrane conductance regulator (CFTR) are multiple-membrane-spanning proteins that are members of the ABC transporter family. For both P-glycoprotein and CFTR, approximately 10–15% of the protein is located in the ER lumen and ER membrane, whereas the bulk is located in the cytosol. The only ER chaperone that interacts with these membrane proteins is calnexin [42, 43]. On the cytosolic side, the most abundant chaperone Hsp70 assists in folding these proteins. Interestingly, when folding attempts fail, Hsp70 also promotes proteasomal degradation of misfolded CFTR by targeting the E3 ubiquitin ligase CHIP to this protein [44]. Cytosolic Hsp90 also has been demonstrated to be involved in degrading CFTR [45]. Since both ER and cytosolic chaperones impose a quality control on this type of membrane protein, it will be of great interest to investigate how events at the two sides of a membrane are communicated and coordinated.

To study a possible interaction of a substrate protein with folding enzymes and chaperones, co-immunoprecipitations can be done using antibodies against folding factors; if interactions are too weak or too transient, chemical cross-linkers may stabilize the complex during analysis. To examine the role of a particular chaperone on the folding of a substrate protein, one may examine the effect of its increased or decreased expression in the cell or its presence or absence in a folding assay. To test whether an ATP-dependent chaperone is involved, ATP can be depleted from cells. Section 3.4 will address these and other conditions that may affect the folding process.

3.3 Location-specific Features in Protein Folding

3.3.1 Translocation and Signal Peptide Cleavage

One location-specific feature of protein folding is its potential coupling to translocation into an organelle and subsequent signal peptide cleavage. Proteins that are targeted to mitochondria, chloroplasts, and the ER normally carry a signal peptide at their N-terminus.

Mitochondria have two membranes: the inner membrane, which encloses the

matrix space, and the outer membrane, which is in contact with the cytosol. The majority of mitochondrial proteins are encoded in the cell nucleus and need to be imported from the cytosol via the macromolecular Tim/Tom complex into the mitochondrial matrix, the mitochondrial inner membrane, or the intermembrane space [46].

Chloroplasts are chlorophyll-containing, double membrane-bounded organelles that are present in all higher plants. They possess the same compartments and membranes as mitochondria but have an extra subcompartment, the thylakoid space, which is surrounded by the thylakoid membrane. Like mitochondria, proteins are posttranslationally imported via the Tic/Toc complex [47].

Proteins that need to be imported into the ER lumen of eukaryotes are translocated from the cytosol through the translocon. Co-translational translocation is signal recognition particle (SRP)-dependent [48]. SRP binds to the signal sequence on the nascent chain, the ribosome/nascent polypeptide/SRP complex docks onto the SRP receptor at the ER membrane, and via multiple steps, the protein is translocated into the ER lumen. Oligosaccharyl transferase (OST) attaches a glycan to the nascent chains, and in most proteins the signal peptide is cleaved off co-translationally by the signal peptidase present in the membrane with its active site in the lumen of the ER. Up to now, only three exceptions have been reported in which signal peptide removal is (at least partially) posttranslational, all of them viral glycoproteins. Posttranslational translocation is SRP-independent; this type of translocation needs additional factors to associate with the translocon complex.

Proteolytic cleavage events in mitochondria, chloroplasts, and the ER can be followed by mobility changes of the protein in reducing SDS-PAGE. If the signal peptide of a certain protein is cleaved off, the protein will have an increased electrophoretic mobility. Signal peptide cleavage in nascent chains is not detectable in a one-dimension gel, but it can be followed in two-dimensional SDS-PAGE, where the protein sample is digested with an SDS-resistant protease between the first and second dimension [49].

To test which population of full-length folding intermediates has lost its signal sequence in the ER (when cleavage is posttranslational), the process can be correlated to disulfide bond formation by using another two-dimensional SDS-PAGE system. Samples are run non-reduced in the first dimension (horizontal) and subsequently are reduced in the second dimension (vertical). In this gel all proteins lacking disulfides will run on the diagonal. If a protein has intrachain disulfide bonds, it will run faster in the second dimension and thus will end up below the diagonal. Proteins that form interchain disulfide-linked complexes will run above the diagonal. Antibodies against the signal peptide itself or antibodies that recognize the N-terminus of the protein after, but not before, cleavage can be used to monitor the process of signal peptide cleavage more directly. Translocation of proteins in a cell-free translation organellar system can be demonstrated by protease-resistance. A newly synthesized protein that is not translocated yet will be degraded by (membrane-impermeable) proteases added to the membranes but will be protected from digestion once translocated.

3.3.2 Glycosylation

Glycan moieties on proteins are essential for folding, sorting, and targeting of glycoproteins through the secretory pathway to various cellular compartments. N-linked glycans affect local conformation of the polypeptide chain they are attached to; increase local solubility of proteins, thereby preventing aggregation; are important for the interaction with the two lectin chaperones in the ER, calnexin and calreticulin; and are important for targeting misfolded proteins for degradation. Hence, for most glycoproteins, their N-linked glycans are indispensable for proper folding.

The glycosylation process starts at the cytosolic side of the ER, where monosaccharides are added to the lipid intermediate dolichol phosphate up to $Man_5GlcNac_2$-PP-Dol. This precursor is translocated to the lumenal side, where it is elongated to $Glc_3Man_9GlcNAc_2$-PP-Dol. The oligosaccharyl transferase then transfers the 14-mer to an asparagine residue in the consensus sequence N-X-S/T of a nascent polypeptide chain, wherein X is any amino acid except proline or aspartic acid. Glucosidase I (GI, a membrane-bound enzyme) removes the first glucose and glucosidase II (GII, a soluble enzyme), the second and third. Monoglucosylated oligosaccharides associate with the ER-resident lectins calnexin (membrane-bound) and calreticulin (soluble), which are in a complex with ERp57 [36]. When GII cleaves the last glucose, the protein is released from calnexin/calreticulin and, if correctly folded, can leave the ER. UDP-Glc:glycoprotein glucosyltransferase (GT), a soluble ER protein of 160 kDa, is a sensor of glycoprotein conformation and reglucosylates glycans close to un- or misfolded amino acid stretches [50–52]. By reglucosylating the protein, it becomes a ligand again for calnexin/calreticulin and therefore a substrate for this quality-control cycle [9]. Two ER mannosidases can remove one or two mannoses in the ER before the protein is transported to the Golgi, where further oligosaccharide processing proceeds and proteins can be *O*-linked glycosylated. Alternatively, a permanently misfolded protein may be targeted for degradation from the ER through glycan recognition (see below).

The enzyme endoglycosidase H (Endo H) often is used to monitor the movement of newly synthesized glycoproteins from the ER to the Golgi complex. Glycans on proteins remain sensitive to digestion by Endo H as long as they are in the ER and in early regions of the Golgi, but they become resistant beyond. Endo H digestion after the immunoprecipitation of a glycoprotein allows conclusions to be made on the protein's location. If a protein is highly heterogeneously glycosylated, e.g., HIV envelope, which has ~30 N-linked glycans, Endo H can also be used after the immunoprecipitation to increase mobility of folding intermediates in the ER and to bring about a collapse of smeary bands into one sharp band in a gel, which enables identification and quantitation. The enzyme PNGase F in principle removes all glycans, irrespective of composition, and theoretically is the better deglycosylation enzyme. Its activity changes the glycosylated asparagine into an as-

partic acid, however, which in some proteins decreases mobility, increases fuzziness of bands, and may confuse the pattern. The enzyme of choice needs to be determined for every protein one studies. To gather more information on how glycosylation affects protein folding, glycosylation site mutants and enzymes involved in glycosylation can be used (see Section 3.4).

3.3.3 Disulfide Bond Formation in the ER

The lumen of the ER supports a relatively oxidizing environment that facilitates the formation of disulfide bonds in folding proteins. Disulfide bonds are thought to stabilize proteins during and after folding, but this is an oversimplification of their role. In the cytosol the ratio between reduced glutathione (GSH) and oxidized glutathione (GSSG) is ~60:1, while in the ER this ratio is 3:1 [53]. Glutathione is the major redox buffer in the ER. Therefore, glutathione was thought for a long time to be responsible for generating and maintaining the redox potential in the ER. This view changed with the identification of the FAD-binding ER oxidoreductase 1 (Ero1p) in *Saccharomyces cerevisiae* and its homologues Ero1α and Ero1β in more complex eukaryotes. Disulfide-linked complexes between Ero1p and PDI have been captured in yeast as well as mammalian cells. In addition, disulfide-linked complexes were found between PDI and a newly synthesized secretory protein [54]. Ero1 transfers disulfides to (or rather, electrons from) substrate proteins via protein disulfide isomerase (PDI). Other oxidoreductases, such as Erv proteins and PDI homologues, play a role as well.

Formation of disulfide bonds in a folding protein can be followed through a pulse-chase assay. In combination with an immunoprecipitation and reducing and non-reducing SDS-PAGE, the formation of disulfides can be monitored (see the Appendix and [11]). To study which enzymes are involved during disulfide bond formation and isomerization, co-immunoprecipitations can be performed with different antibodies against folding enzymes with or without prior cross-linking. Furthermore, the folding enzyme concentration can be changed, as is described below.

If the studied protein contains disulfide bonds, an alkylating agent must always be added to the stop solution and lysis buffer of the pulse-chase assay to block free sulfhydryl groups [55, 56]. This will prevent formation of (additional) disulfide bonds in the protein after cell lysis. Three commonly used blocking agents are iodoacetamide (IAM), iodoacetic acid (IAC), and *N*-ethyl maleimide (NEM). IAM and IAC bind relatively slowly, but irreversibly. IAC is negatively charged, which decreases mobility of proteins it is attached to. NEM is smaller than IAM and IAC; it binds fast but is under certain conditions partially reversible. It is therefore less suitable when prolonged incubation periods at 37 °C are required. Since all alkylating agents have different characteristics, the alkylation protocol may be optimized for each individual protein.

3.3.4 Degradation

In the ER, a stringent quality-control system operates that discriminates between correctly folded proteins, misfolded proteins, and unassembled protein subunits. This prevents misfolded proteins from leaving the ER, where they can cause harm to the cell or even to the complete organism. The ER-associated degradation (ERAD) pathway ensures ubiquitin-mediated degradation of ER-associated misfolded proteins [57]. Trimming of the α1,2-linked mannose of the middle branch by mannosidase I to $Man_8GlcNac_2$ serves as degradation signal. EDEM (ER degradation-enhancing α-mannosidase-like protein) functions in the ERAD pathway by accepting terminally misfolded glycoproteins from calnexin [58, 59]. The substrate is retro-translocated into the cytosol, where proteins are deglycosylated, ubiquitinated, and then degraded by the 26S proteasome [57]. Post-ER quality-control systems exist as well: proteins can be transported to the Golgi or the plasma membrane before they are degraded, most often by vacuolar/lysosomal proteases.

Proteins can be degraded after synthesis as well as during their translation, which is undetectable in a pulse-chase assay. The net result is a lower amount of labeled protein, which can be visualized only through the use of a proteasome inhibitor during the pulse labeling. If the total signal of labeled protein at the end of the pulse is higher in the presence than in the absence of the inhibitor, co-translational degradation apparently does happen in the control situation. Degradation *after* translation can be followed on gel, because the total protein signal will decrease during the chase. Additionally, proteasome inhibitors and lysosomal enzyme inhibitors can be added to prove degradation and identify the site of breakdown. A useful system to study protein folding without degradation is an in vitro translation system in the absence or presence of a membrane source, the latter ranging from enriched organelles to digitonin-permeabilized, semi-intact cells. The hemin present in the reticulocyte lysate inhibits proteasomal activity.

3.3.5 Transport from ER to Golgi and Plasma Membrane

Different methods can be used to monitor transport of a protein out of the ER to the Golgi complex or the plasma membrane. Endo H resistance is a good tool to monitor the movement of newly synthesized glycoproteins from ER (sensitive to digestion by Endo H) to Golgi (resistant to Endo H). Resistance of newly synthesized proteins to reduction by DTT (or oxidation by diamide) can be used to determine the folding status of a protein. If a protein is transported very rapidly from ER to Golgi, which then coincides with rapid and massive changes in electrophoretic mobility, such as *O*-glycosylation [60] or *N*-glycan modification [61], and one wants to trap the protein in the ER, in vitro translation in the presence of mi-

crosomes or semi-intact cells can be used, since these systems lack ER-to-Golgi transport.

Cell surface arrival can be shown using protease digestion on intact cells or biotinylation. Immunofluorescence can be used to show a plasma membrane staining or to follow the traveling protein with time throughout the cell, provided that it is fused to the fluorescent GFP marker protein, and only when conditions are used in which the studied processes can be synchronized, such as the release from a synthesis block (cycloheximide incubation and removal), a disulfide-formation block (DTT incubation and removal), or a conditional mutant, often used in *S. cerevisiae*. Indirect immunofluorescence allows detection of possible locations of a protein in the cell, but this steady-state method does not allow any conclusions to be made on precursor-product status or on kinetics of processes. Receptor or ligand binding can be tested, and if the studies concern a soluble protein, protein secretion can be measured by collecting the chase medium.

3.4 How to Manipulate Protein Folding

3.4.1 Pharmacological Intervention (Low-molecular-weight Reagents)

To study the role of folding factors in vivo, their activity needs to be manipulated. Drugs act fast and do not allow time for genetic adaptation of cells, but they may not inhibit to 100% and may exhibit pleiotropic effects. Genetic changes of cells circumvent this lack of specificity. One particular folding factor can be changed, but compensatory regulation processes may completely change the protein composition of the cell. With these limitations in mind, both high- and low-molecular-weight manipulations can increase our understanding of protein folding in cells.

3.4.1.1 Reducing and Oxidizing Agents

Reducing and oxidizing agents not only are useful to determine the conformation of a folding protein (as discussed in Section 3.2), but also they can be used as a tool to manipulate protein folding. If separation of protein translation and protein folding is desired, for example, to study protein folding in the absence of ATP or to determine whether co-translational disulfide bond formation is needed for a certain protein to fold, a reducing agent such as DTT can be added to the pulse medium to prevent disulfide bond formation during protein synthesis. Furthermore, reducing and oxidizing agents can be added to the chase medium to determine the effect of reduction/oxidation on protein folding and (posttranslational) signal peptide cleavage.

3.4.1.2 Calcium Depletion

In addition to its role in protein folding, the ER has an important role in calcium signaling and in sequestering calcium from the cytosol. The ER contains

many calcium-binding proteins, which have a large capacity for calcium and help keep the cellular internal calcium concentration constant. The first identified calcium-binding protein was calsequestrin in the sarcoplasmic reticulum of striated muscle [62]. Its C-terminal domain binds calcium with low affinity but with high capacity. The ER protein calreticulin predominates in most non-muscle cells. The calcium level in the ER is important not only for signaling but also for proteins traveling through the ER. During folding they are assisted by calcium-binding molecular chaperones such as BiP (Grp78), Grp94 (endoplasmin), and the lectins calnexin and calreticulin. When the level of calcium in the ER drops, some of the newly synthesized proteins misfold and aggregate [63]. To study the effect of calcium on protein folding, calcium in the cell can be depleted using various chemicals. A23187 is a divalent cation ionophore [64] that can be added to calcium-free starvation, pulse, and chase media. Thapsigargin is a potent cell permeable, IP3-independent, intracellular calcium releaser, as it efficiently inhibits all members of the Ca-ATPase family of calcium pumps, which pump calcium into the ER [65, 66]. Calcium chelators such as BAPTA, EDTA, and EGTA can be used as well to study the effect of calcium (and calcium-binding chaperones) on protein folding.

3.4.1.3 **ATP Depletion**

ATP is a common currency of energy in all cells, and its presence is needed for various reactions and processes, including protein folding [67]. The ER contains many chaperones such as BiP, the lumenal Hsp70 of the ER, which has an N-terminal ATP-binding domain and a C-terminal peptide-binding domain and whose activities are tightly coupled. In the Hsp70 family of chaperones, association and dissociation with the substrate protein are controlled by ATP binding and hydrolysis. To test the effect of ATP(-dependent chaperones) on protein folding, ATP generation can be blocked. 2-Deoxy-glucose inhibits glycolysis and, thereby, anaerobic ATP generation. It can be added to glucose-free cell culture medium. Sodium azide poisons mitochondria and thus inhibits aerobic ATP generation. Combination of both treatments usually depletes ATP effectively from living cells [67]. After depletion, ATP levels in the cell lysates can be measured, e.g., with a luciferase-luciferin assay in a scintillation counter or luminometer [21, 68].

3.4.1.4 **Cross-linking**

Chemical cross-linkers are useful for multiple purposes in protein science, such as the stabilization of protein-chaperone complexes. There are two prominent groups of cross-linkers: homobifunctional cross-linking reagents, which have two identical reactive groups, and heterobifunctional cross-linking reagents, in which the reactive groups are chemically distinct. Further variation is present in spacer arm length, cleavability, or membrane permeability, and cross-linkers may react chemically or photochemically upon UV illumination. Whereas membrane-permeable cross-linkers are suitable for cross-linking in the cell, membrane-impermeable reagents are useful for cross-linking at the cell surface and in cell lysates. Cross-linkers with shorter spacer arms (4–8 Å) often are used for intramolecular

cross-linking, and reagents with longer spacers are favored for intermolecular cross-linking. The most frequently used cross-linkers at the moment are DSP (dithio-*bis*-succinimidylpropionate), a water-insoluble, homobifunctional *N*-hydroxysuccimide ester with a spacer arm length of 12 Å, which is thiol-cleavable and primary amine reactive and is used in many applications [69], and BMH (*bis*-maleimidohexane), a water insoluble, homobifunctional, non-cleavable cross-linker with a spacer arm length of 16 Å, which is reactive towards sulfhydryl groups and is also used in many applications [70, 71]. Since there is a wide variety in characteristics and potential applications of cross-linkers, it is desirable to compare different cross-linkers before protein-folding studies are performed.

3.4.1.5 Glycosylation Inhibitors

Inhibitors that prevent glycan synthesis or modify the carbohydrate portion of the glycoprotein are useful to determine function of the glycan or change fate of a protein. A wide variety in glycosylation inhibitors exists, each blocking a specific step in glycosylation. Tunicamycin, for instance, was isolated from *Streptomyces lysosuperificus* in the early 1970s [72]; it inhibits transfer of *N*-acetylglucosamine to dolichol phosphate, thereby completely blocking N-linked glycosylation, which most often causes misfolding of glycoproteins. The misfolding of many proteins in the ER can lead to upregulation of classical chaperones and folding-facilitating proteins through a so-called unfolded protein response (UPR) [73].

In order to avoid the complete inhibition caused by tunicamycin, which can trigger the UPR, more subtle inhibitors can be used, e.g., castanospermine. This is a plant alkaloid that was isolated from the seeds of the Australian tree *Castanospermum australe*, which inhibits glucosidase I and II. An example of a glucosidase I-specific inhibitor is australine, whereas deoxynojirimycin inhibits glucosidase II better than glucosidase I. Glucosidase I and II inhibitors can be useful in preventing association of the folding protein with the molecular chaperones calnexin and calreticulin, which bind only to monoglucosylated proteins. If a prolonged association with calnexin or calreticulin is desired, a glucosidase II inhibitor can be added during the chase period in a pulse-chase assay. This prevents cleavage of the last glucose, such that the glycoprotein will remain bound to the lectins calnexin and calreticulin. If a mannosidase I inhibitor such as kifunensine is added during the pulse chase, degradation of misfolded glycoproteins can be prevented.

Glycosylation inhibitors are useful reagents in determining the role of oligosaccharides during protein folding, but because they often do not inhibit 100% and because some inhibitors can have an effect on protein synthesis, it is very important to always include the proper controls when they are used in experiments.

3.4.2 Genetic Modifications (High-molecular-weight Manipulations)

3.4.2.1 Substrate Protein Mutants

To study the effect of certain amino acids or modifications on the folding pattern of a protein, various mutations can be made in the cDNA encoding the protein.

To investigate the effect of glycosylation on a protein, *N*-glycosylation consensus sequences can be removed or created by site-directed mutagenesis. Mutations of choice are the exchange of Asn for Gln or the downstream Ser or Thr for Ala; to exclude the effect of an amino acid change rather than of a lacking glycan, both strategies can be used and compared. This allows study of the role of every individual glycosylation site for proper folding, alone or in combination. Additional glycosylation sites can be generated to study the effect of hyperglycosylation. The addition of glycosylation consensus sequences is also used to determine the topology of a protein; interpretation of such data needs care, however, because a glycan may change the topology or fate of a protein.

The role of a specific disulfide bond for protein folding can be studied by changing the cysteines into another residue using site-directed mutagenesis. By creating single-cysteine and double (-cystine) mutations, the effect of removal of a complete disulfide bond on protein folding can be compared to the removal of a single cysteine, hence the presence of an odd number of cysteines. Cysteines are best changed into alanines, because they leave ''only'' a small gap in the protein structure, i.e., where the thiol atom resided. Although serines are thought to resemble cysteines more closely than alanines, their higher hydrophilicity and their hydrogen atoms may cause problems when incorporated into the tightly packed core of a protein. Methionines and valines in some instances are better replacements for cysteines, but they may be too large in other sites. To be certain that the result reflects the removal of a disulfide bond rather than the change of an amino acid in the sequence, it is wise to substitute cysteine for more than one other amino acid and compare the effects.

Many other mutants can be thought of. The signal peptide of a protein can be modified to determine which residues are responsible for signal peptide cleavage, and the cleavage site can be changed to determine the effect of timing and cleavage on folding. In addition, the original signal sequence can be replaced completely by the signal sequence of another protein, and folding kinetics and secretion can be studied, as was done for HIV envelope [74]. To determine sequence and hierarchy of domain folding within a protein, and to determine whether domains fold independently of each other, single domains can be expressed, and C-terminal and N-terminal truncation mutants can be studied.

When protein mutations result in clinical phenotypes, as in many inherited diseases, it is interesting to focus on those mutations that result in folding defects.

4.4.2.2 **Changing the Concentration or Activity of Folding Enzymes and Chaperones**

To determine the effect of molecular chaperones and folding enzymes on the folding of a protein, the protein can be overexpressed by infection/transfection. Cells can be transiently transfected using a plasmid encoding the protein of interest behind an appropriate promoter. High expression can be achieved using virus-based expression systems; one example is the recombinant vaccinia virus expressing T7 polymerase in combination with a plasmid containing the protein of interest behind the T7 promoter. Stable, overexpressing cell lines can be used as well; one ex-

ample is the Dorner cell lines [75], which overexpress a particular ER chaperone, such as PDI, BiP, or Grp94.

Because of the redundancy of many chaperones and folding enzymes, overexpression of a folding factor may not lead to a desired effect. Lowering expression or activity may be more successful. In cases where molecular details of the activity of a folding factor are known, dominant-negative mutants have been generated: the "trap" mutants of chaperones such as GroEL [76] and BiP [77] and active-site mutants of enzymes such as Ero [78]. Many cell lines exist in which a particular folding factor is absent or incapacitated. Examples are the CHO-derived Lec cell lines [79], which include the glucosidase I-negative Lec23 cells [80]. Other examples are the BW 5147 mouse lymphoma-derived PhaR2.7 cell line, which is glucosidase II-deficient (because of which the lectins calnexin and calreticulin cannot bind to their substrates anymore) [81], and the human T lymphoblastoid cell line CEM.NKR [82], which is resistant to natural killer cells (NK cells) and has no calnexin.

The most dramatic effect can be expected when a folding factor is completely absent. In various organisms such as yeast, genes can be deleted. In mammalian cell lines, this is still a procedure with little success. Alternatives are cell lines derived from knockout animals, which are becoming increasingly available. In other organisms or cell lines, depletion of a gene is the maximum reachable. Gene expression can be suppressed by siRNA, which was first discovered in the nematode worm *C. elegans*; this animal is capable of sequence-specific gene silencing in response to double-stranded RNA (dsRNA) [83]. The technology has been adapted for use in mammalian cells, and an increasing number of siRNA constructs and libraries are becoming available, which makes siRNA a powerful tool to study how chaperones and folding enzymes influence protein folding.

3.5 Experimental Protocols

3.5.1 Protein-labeling Protocols

3.5.1.1 Basic Protocol Pulse Chase: Adherent Cells

In vivo, conformational changes during folding, disulfide bond formation, signal peptide cleavage, other proteolytic processing steps, glycosylation and glycan modifications, transport to other compartments, and some aspects of biological activity all can be studied in intact suspension cells or in adherent cells growing in monolayers in tissue culture dishes [11]. The adherent cells are easiest to use because they allow multiple wash steps in a short period of time, which are needed for short pulse and chase times or for studies requiring frequent changes of media with very different composition. When cells do not adhere or when low volumes of media are desirable (e.g., when expensive additives are needed), most of the analyses can be done in suspension, as described in the alternate protocol. Prepare

the cells expressing the protein of choice in 35- or 60-mm dishes. For each time point, at least one dish is needed; the protocol is written for 60-mm dishes. On the day of the pulse chase, the cell monolayer needs to be almost confluent (80–90%).

Prepare all solutions needed. Also prepare a pulse-chase scheme on which all steps in the procedure (pulse, chase, stop) are noted. We use a 1-min scheme, meaning that every minute one action (pulse, chase, or stop) is performed. If aspiration is fast (which depends on the vacuum system used) and the experimenter is experienced, 30-s intervals become possible.

1. Prepare the pulse-chase setup, which includes a water bath at the desired temperature, with tube racks and 1–2 mm water above them, which can be maintained at this temperature. *Make sure the water level is just above the racks, such that the bottom of a tissue culture dish is completely in contact with the water but dishes are not floating when they are without cover.*
2. Wash the cells with 2 mL wash buffer and add 2 mL starvation medium. Incubate for 15–40 min. at 37 °C in the presence of 5% CO_2.
3. Place the dishes on the rack in the water bath. *Make sure there are no air bubbles below the dishes.*
4. After 15 min pre-incubation with starvation medium, start the first pulse labeling. The staggering schedule (Figure 3.2) should allow labeling of the last dish within ∼40 min of the start of starvation. Pulse label the cells one dish at a time: aspirate depletion medium, add 400 μL labeling medium at time 0 s. to the center of the dish, swirl gently, and incubate for the desired labeling time on the water bath. For pulse times longer than 15 min, a larger volume and incubation (on a rocker) in a 37 °C incubator in the presence of 5% CO_2 is recommended. *The pulse time optimally is equal to or shorter than the synthesis time of the protein under study (an average of 4–5 amino acid residues per second). Labeling time should be long enough to detect the protein.*
5. Add 2 mL chase medium at precisely the end of the pulse (i.e., at time 2′0″ if a 2-min pulse is aimed for). Swirl gently to mix; the labeling stops immediately upon addition of chase medium. Aspirate this mix of labeling and chase medium and add another 2 mL of chase medium. For short chases: incubate on the water bath. For chases longer than 30 min, incubate dishes in the incubator. For 0′ chase: add 2 mL chase medium at precisely the end of the pulse. Swirl gently to mix. Aspirate chase medium, place the dish immediately on ice, and add 2 mL of ice-cold stop buffer. *This is best achieved by putting an aluminum plate on top of an ice pan full of ice, with a wet paper towel on top to allow optimal contact between the plastic of the cell culture dish and the ice-cold surface.*
6. Stop the chase: aspirate chase medium at precisely the endpoint of the chase time, place the dish on the aluminum plate on ice, and immediately add 2 mL of ice-cold stop buffer. *If the studied protein will be secreted, collect the chase medium and use it for immunoprecipitation.*
7. Just before lysis: wash the cells again with 2 mL ice-cold stop buffer.

8. Aspirate the dish as dry as possible without letting cells warm up and add 600 µL ice-cold lysis buffer.
9. Scrape the cell lysate and nuclei off the dish with a cell scraper and transfer the lysate to a microcentrifuge tube.
10. Centrifuge lysates at 16 000 *g* at 4 °C to spin down the nuclei. Use the supernatant directly for immunoprecipitations or transfer the supernatant to a new microcentrifuge tube, snap freeze, and store at −80 °C.

Buffers for the Pulse Chase

Wash buffer: Hank's Balanced Salt Solution (Invitrogen) or PBS with calcium and magnesium. Keep at 37 °C.

Starvation medium: cysteine- and methionine-free tissue culture medium (ICN) supplemented with 10 mM Na-HEPES (pH 7.4). Keep at 37 °C.

Pulse medium: cysteine- and methionine-free medium containing 10 mM Na-HEPES (pH 7.4), 125 µCi ^{35}S-cysteine/methionine per milliliter. Keep at 37 °C. *To manipulate protein folding, chemicals such as glycosylation inhibitors, proteasome inhibitors, and DTT can be added to the pulse medium.*

Chase medium: complete tissue culture medium for the appropriate cell line (also a 1/1 mix with serum-free medium is possible) supplemented with 10 mM Na-HEPES (pH 7.4), 5 mM cysteine, and 5 mM methionine. Keep at 37 °C. *If protein-folding kinetics is to be studied, 1 mM cycloheximide needs to be added to the chase medium to stop elongation of unfinished nascent chains; without it, the amount of labeled full-length protein may increase during the chase, even though incorporation of ^{35}S label is stopped completely. To manipulate protein folding, chemicals such as glycosylation inhibitors, proteasome inhibitors, DTT, diamide, or cross-linkers can be added to the chase medium, or ATP can be depleted.*

Stop buffer: Hank's Balanced Salt Solution (Invitrogen) or PBS with calcium and magnesium, supplemented with 20 mM alkylating agent (IAC, IAM, or NEM). Keep at 0 °C. *See Section 3.4.*

Lysis buffer: PBS (pH 7.4) or similar salt-containing buffer, containing 0.5% Triton X-100, 1 mM EDTA, 20 mM alkylating agent, 1 mM PMSF, and 10 µg µL^{-1} each of chymostatin, leupeptin, antipain, and pepstatin. Keep at 0 °C. *Add PMSF to the lysis buffer just before lysis. PMSF is highly unstable in water and has a half-life of only a few hours on ice. Different proteins may need the use of different protease inhibitors. The combination in the protocol is an empirically determined one that works well for proteins used in our lab. Salt concentration should be isotonic to prevent nuclei from disrupting. When noncovalent interactions are studied, other detergents may work better. Examples include CHAPS, octyl glucoside, or digitonin. When the protein associates with detergent-resistant membranes, the use of Triton X-100 and CHAPS should be avoided. Octyl glucoside is one of the detergents that will solubilize all membranes.*

Postponed Posttranslational Folding To separate translation from the folding process, e.g., to study the effect of factors that would affect translation, such as ATP

depletion, oxidative folding can be postponed until after synthesis by addition of a reducing agent to the pulse medium [20]. This will prevent disulfide bond formation during the pulse. When the reducing agent is removed after the pulse, disulfide bond formation and folding can proceed. For various proteins, oxidative folding can be postponed until after synthesis without it creating a problem for folding.

1. Add DTT to the pulse medium to a concentration of 5–10 mM and pre-incubate for 5 min with DTT before the pulse. *The DTT concentration needs to be titrated for each protein, but usually 5 mM should be sufficient. Since labeling efficiency might decrease in DTT, labeling time may need to be increased. DTT may affect cellular ATP levels during long incubations, depending on the cell line. Therefore, a comparison with co-translational folding always needs to be made and ATP levels need to be measured.*

DTT Resistance When proteins fold and become more compact, this is usually reflected by resistance to reduction by DTT in vivo. Influenza hemagglutinin, for example, acquires DTT resistance when it is a completely oxidized monomer that is trimerization-competent but not yet a trimer [21].

1. Label the protein (see pulse-chase protocol) and chase for different times.
2. After the chase: perform in a parallel dish with the same chase time an additional chase of 5 min with 5 mM DTT. Compare this sample with the sample in which the protein is chased without DTT.

Diamide Resistance

1. Label the protein (see pulse-chase protocol) and chase for different times.
2. After the chase: perform in a parallel dish with the same chase time an additional chase of 5 min with 5 mM diamide. Compare this sample with the sample in which the protein is chased without diamide.

3.5.1.2 Pulse Chase in Suspension Cells

When cells do not adhere properly or when small volumes are desired, cells can be pulse labeled in solution. In this assay the different chase samples can be collected from one tube. Wash steps after the pulse and chase are not included, because the centrifugation steps would take too much time. Prior to starting the experiment it is necessary to determine:

- the minimum volume for incubating the cells during the pulse (x µL), (*This is cell line dependent.*)
- the number of chase points (y), and
- the desired sample volume (z). We used, for example, $x = 300$ µL, $y = 3$, and $z = 500$ µL.

1. Transfer suspension cells to a sterile 50-mL tube with cap. We used, for example, $\sim 10^6$ cells per time point.
2. Pellet cells at 500 *g* for 4 min at RT. Resuspend cells in $2y$ mL depletion medium and pellet cells again. Resuspend cells in $2y$ mL depletion medium. Incubate cells for 15 min at 37 °C in a CO_2 incubator.
3. Pellet cells at 500 *g* for 4 min at RT. Resuspend cells in x µL depletion medium, change to appropriate tube if necessary, and place in a water bath at 37 °C or the desired temperature for the experiment.
4. Add 50–100 µCi ^{35}S-labeled methionine and cysteine per time point and mix gently to start the pulse. Incubate for the pulse period.
5. Add $\geq yz$ µL chase medium. The total volume should be slightly more than $y \times z$ µL to allow for fluid loss due to evaporation during the experiment. Mix by gently pipetting up and down.
6. Immediately take the first sample (z µL). Transfer to microcentrifuge tube with pre-prepared z µL 2× concentrated lysis buffer on ice, mix well, and keep on ice.
7. After every chase interval, collect a sample and add to 2× concentrated lysis buffer on ice, mix well, and keep on ice.
8. Spin the lysates at 16 000 *g* for 10 min at 4 °C to pellet the nuclei. The postnuclear lysate can be directly used for immunoprecipitations or transferred to a new microcentrifuge tube, snap frozen, and stored at −80 °C.

Semi-intact Cell System (see Chapter 18) In vitro translation in the presence of semi-intact cells [12] is a method with a complexity between the pulse-chase assay and the in vitro chase assay, since there is only one membrane between the protein and the experimenter, whereas there are two in the pulse-chase assay and none during the second part of the in vitro chase assay.

In the semi-intact cell system, cells are treated with the detergent digitonin, which selectively permeabilizes the plasma membrane, leaving cellular organelles such as ER and Golgi complex intact. These semi-permeabilized cells (SP cells) are added to an in vitro translation system, where they act as a source of ER membranes. Only the mRNA of the protein of interest is present, which precludes the need for immunoprecipitations. This assay is especially useful when there are no antibodies available against the protein under study, for protease resistance studies, or when only the ER form of a protein is under study and ER-to-Golgi transport is undesirable.

In Vitro Chase Assay In the in vitro chase assay (Maggioni et al., in press), a protein is pulse labeled/translated in vivo and chased in a detergent lysate. Since there are no membranes during the chase period, the direct environment of the folding protein can be manipulated. For example, ATP levels in the detergent lysate can be depleted, and chaperones and organellar cell fractions can be added.

After point 4 of the basic pulse-chase protocol (now using 10-cm dishes):

For the in vitro assay 10-cm dishes are used instead of 6-cm dishes. Therefore, all buffer volumes used in steps 1–4 of the basic pulse-chase protocol need to be multiplied by 2–2.5.

1. Wash cells twice with 4 mL ice-cold HBSS wash buffer, aspirate the buffer, and immediately lyse with 1.8 mL ice-cold lysis buffer without alkylating agent.
2. Scrape the cell lysate and nuclei off the dish with a cell scraper, transfer lysates to a microcentrifuge tube, and immediately take out 1/6 of the lysate and add NEM to this sample to a concentration of 20 mM (0 min sample in the in vitro chase assay).
3. Spin cell lysates for 10 min at 16 000 *g* at 4 °C to pellet nuclei. Transfer post-nuclear cell lysate to a new tube. The supernatant of the lysate without NEM is used in the rest of the in vitro chase assay.

After transfer to a new tube, add 5 mM GSSG and transfer the tube immediately to a water bath at 30 °C.

4. After different times of in vitro chase, take 300 µL of the lysate and transfer it into a new tube that contains NEM to a final concentration of 20 mM and immediately incubate on ice.
5. Use the supernatant directly for immunoprecipitations or snap freeze and store at −80 °C.

3.5.2 (Co)-immunoprecipitation and Excessary Protocols

3.5.2.1 Immunoprecipitation

1. Mix 50 µL of washed protein A-Sepharose beads and the optimal amount of antibody and shake in a shaker for 60 min at 4 °C. *Optimize the incubation time for each antibody. The optimal amount of antibody is the amount that precipitates all, or at least the maximum amount, of antigen from the solution. This needs to be tested for each antibody-antigen combination by re-incubation of the supernatant from the beads-antibody-antigen incubation (see step 3 below) with antibody and beads. If the antibody has a low affinity for protein A, protein G-Sepharose beads or a bridging antibody between the primary antibody and the protein A beads, such as a goat or rabbit anti-mouse Ig, can be used. Instead of Sepharose beads, heat-killed and fixed* S. aureus *cells can be used to immobilize the antibody. Because these cells are more difficult to resuspend than Sepharose beads, spin conditions should be 5 min at 1500* g *rather than 1 min at max speed. Resuspension time will be longer and should be added to the wash time.*
2. Add 100 µL to 600 µL post-nuclear cell lysate and couple in the head-over-head rotator for at least 30 min (depending on the antibody) at 4 °C. *Test for each antibody the minimum incubation time needed (between 30 min and overnight). With one lysate, different immunoprecipitations (such as one with an antibody that recognizes all forms of the protein, a conformation-sensitive antibody, or a co-immunoprecipitation) can be done to obtain as much information as possible on the folding protein.*
3. Pellet beads by spinning 1 min at RT max, aspirate the supernatant, and use it for re-precipitation to test whether the antibody precipitated all antigen molecules in the solution (see step 1 above). Add 1 mL washing buffer and shake

for at least 5 min in a shaker at RT. Repeat this washing procedure once. *Test different wash buffers with different concentrations of SDS, other detergents, combinations thereof, and salt. Also test different wash times and wash temperatures. It is not necessary to wash more than twice. Rather than increase the number of washes, conditions of the two washes should be changed. If this does not give the desired result, pre-clearing of the detergent cell lysate is the method of choice (see calnexin protocol).*

4. Pellet beads and aspirate supernatant. Add 20 µL TE to the beads, vortex to resuspend the beads, and add 20 µL 2× concentrated sample buffer and vortex again. *If an Endo H digestion is performed, add here the Endo H buffer instead of the TE. It is important to resuspend in TE before adding SDS, because addition of SDS to a pellet may induce or increase aggregation.*
5. Heat samples for 5 min at 95 °C, vortex when still hot, and pellet the beads. The supernatant is the non-reduced sample.
6. To make a reduced sample, transfer 18 µL supernatant to a new tube to which a defined drop of DTT solution has been added (final DTT concentration should be 20 mM) and vortex. Heat samples for 5 min at 95 °C. Centrifuge shortly to spin down the condensation fluid. The samples are now ready to be loaded onto an SDS-PA gel. *Addition of DTT to the bottom of the tube allows one to check whether DTT actually has been added to the sample. Re-oxidation during electrophoresis, which happens if proteins have many cysteines, is prevented by adding an excess alkylating agent, such as 100 mM NEM, to all samples before loading.*

Buffers for Immunoprecipitation

Protein A-Sepharose beads: 10% protein A-Sepharose beads in PBS or the same buffer in which the cells were lysed in, supplemented with 1/10 of the concentration of the same detergent used in the lysis buffer and 0.25% BSA. *The beads are washed twice in this solution to remove the buffer in which the Sepharose was stored. When using* S. aureus *cells, washing should be done twice as well, in the same centrifuge that will be used for the immunoprecipitation, to ensure that the same-sized particles will be pelleted during all washes.*

Wash buffer: PBS, pH 7.4, containing 0.5% Triton X-100 or 150 mM NaCl (representing some of the mildest conditions) or another buffer (such as a harsher one with SDS).

TE buffer: 10 mM Tris-HCl, pH 6.8, 1 mM EDTA.

2× concentrated sample buffer: 400 mM Tris-HCl, pH 6.8, 6% (w/v) SDS, 20% glycerol, 2 mM EDTA, 0.04% (w/v) bromophenol blue.

5× concentrated sample buffer: 1 M Tris-HCl, pH 6.8, 7.5% (w/v) SDS, 50% glycerol, 5 mM EDTA, 0.08% (w/v) bromophenol blue.

3.5.2.2 Co-precipitation with Calnexin ([84]; adapted from Ou et al. [85])

After step 7 from the pulse chase:

1. Aspirate the dish as dry as possible and add 600 µL ice-cold lysis buffer (2%

CHAPS in 50 mM Na-HEPES pH 7.6 and 200 mM NaCl. *Do not add EDTA to the lysis buffer, since calcium is needed for the interaction.*

2. Scrape the cell lysate and nuclei from the dish and transfer into a microcentrifuge tube.
3. Use around 200 μL of the lysate for the co-immunoprecipitation and use a parallel amount for an immunoprecipitation using an antibody that recognizes all antigen that is present.
4. Add 0.1 mL of a 10% suspension of heat-killed and fixed *S. aureus* cells to the lysate to reduce background and rotate or shake for 30–60 min at 4 °C.
5. Pellet the fixed *S. aureus* and nuclei at max speed to remove the smallest particles and use the supernatant for the immunoprecipitation.
6. Couple the antibody to the protein A-Sepharose beads for 30 min at 4 °C in a shaker.
7. Add the pre-cleared lysate.
8. Couple at 4 °C for 1–16 h, depending on the antibody.
9. Pellet the beads by centrifugating for 2 min at 8000 *g*.
10. Wash twice with 0.5% CHAPS in 50 mM Na-HEPES pH 7.6, containing 200 mM NaCl.
11. After the last wash: continue the immunoprecipitation protocol at step 4.

3.5.2.3 Co-immunoprecipitation with Other Chaperones

The calnexin co-immunoprecipitation protocol can be used as a basic protocol when the interaction of a protein with another chaperone is to be studied. Since each protein-chaperone interaction is different, protocols should be optimized for each new co-immunoprecipitation. To stabilize the interaction of the molecular chaperone BiP with its substrate, adding an ATP-depleting agent to the lysis buffer is recommended, to lock the ATP cycle of the chaperone. When interactions with calcium-binding chaperones are studied, calcium chelators such as EDTA should be omitted from the lysis buffer.

3.5.2.4 Protease Resistance

Limited proteolysis can be used to investigate the conformation of proteins. However, most investigators often perform limited proteolysis on purified protein, which will represent only the steady-state conformation. To obtain more dynamic information on how proteins acquire their conformation during the folding process, one can combine in vivo pulse-chase analysis and immunoprecipitation, as described earlier in this chapter, with limited proteolysis. Important considerations should be made before using this technique. First, methionines and cysteines within the protein should be evenly distributed. Second, a highly specific antibody is needed to immunoprecipitate the protein of interest. Third, a mild denaturing buffer should be used for cell lysis and immunoprecipitation to prevent large conformational changes of the protein. For most proteins in our lab, we use MNT (20 mM MES, 100 mM NaCl, 30 mM Tris-HCl, pH 7.4) containing 0.5% Triton X-100 as a mild lysis buffer. It is important to omit protease inhibitors and EDTA during cell lysis. Therefore, perform the immunoprecipitation as shortly as possible to prevent protein degradation.

After the last wash of the immunoprecipitation protocol (step 3):

(Note: one protease concentration tested in a proteolysis experiment is one immunoprecipitation.)

1. Pellet the antigen-antibody-bead complexes and aspirate all wash buffer.
2. Add 10 μL MNT + 0.5% Triton X-100 to the beads and resuspend gently.
3. Add 1 μL protease from a 10× stock. Resuspend by vortexing and incubate for *exactly* 15 min on ice. Use a protease titration range (for example, 0.25, 1, 5, 25, 100, or 500 μg mL^{-1} final concentration) to investigate protease susceptibility of each protein. TPCK-trypsin, TLCK-chymotrypsin, proteinase K, and endoproteinase Glu-C V8 are suitable proteases (Sigma) for these studies.
4. After incubation, inhibit the protease by first adding 1 μL PMSF (phenylmethylsulfonyl fluoride) from a 10× stock before adding 10 μL 2× sample buffer. TPCK-trypsin is specifically inhibited by adding a fivefold excess of soybean trypsin inhibitor.
5. Heating the sample for 5 min at 95 °C will completely inactivate the protease.
6. Analyze the radio-labeled proteolytic pattern on a 12–15% SDS-PA gel and expose to film or phosphor screen.

3.5.2.5 **Endo H Resistance**

After the last wash of the immunoprecipitation protocol (step 3):

1. Pellet beads and aspirate all wash buffer from the protein A-Sepharose beads.
2. Add 15 μL 0.2% SDS in 100 mM sodium acetate, pH 5.5, and heat for 5 min at 95 °C to denature the protein.
3. Cool and add 15 μL 100-mM sodium acetate, pH 5.5, containing 2% Triton X-100 and protease inhibitors: 10 μg μL^{-1} each of chymostatin, leupeptin, antipain, and pepstatin and 1 mM PMSF. Add 0.0025 U Endo H (Roche).
4. Mix and incubate for 1.5–2 h at 37 °C. *Optimize incubation time for each protein.*
5. Spin down the fluid and add 7.5 μL 5× sample buffer and mix.
6. Heat for 5 min to 95 °C.
7. Spin down. At this moment a reducing sample can be prepared (see basic pulse-chase protocol).

3.5.2.6 **Cell Surface Expression Tested by Protease**

Arrival of a protein that followed the secretory pathway at the plasma membrane can be biochemically monitored. At the cell surface, the protein is accessible to various reagents, such as antibodies, proteases, and biotinylation agents. Below is a typical protocol for cell surface detection by proteases.

1. Wash the cells twice with 2 mL stop buffer.
2. Add 0.5 mL PBS containing 100 μg mL^{-1} trypsin or another protease and 2 mM $CaCl_2$ (if the protease needs calcium) at 4 °C to the cells.
3. Incubate for 30 min on ice. *At this step surface accessible protein either will be completely digested, or it will be cleaved specifically into a limited number of fragments.*

4. Collect fluid from cells and add 100 μg mL^{-1} soybean trypsin inhibitor, 1 mM PMSF, and 10 μg mL^{-1} each of chymostatin, leupeptin, antipain, and pepstatin (final concentrations).
5. Add to the cells 0.5 mL of 100 μg mL^{-1} soybean trypsin inhibitor, 1 mM PMSF, and 10 μg mL^{-1} each of chymostatin, leupeptin, antipain, and pepstatin (final concentrations). Incubate on ice and repeat the incubation. *It is essential to properly inhibit the protease, because it will otherwise digest internal protein upon lysis.*
6. Lyse the cells in 600 μL lysis buffer and continue with the pulse-chase protocol at step 9. *The comparison between untreated and treated cells will show how much of the protein of interest disappeared because of the proteolysis, or the treated sample will contain a mixture of undigested (internal) and digested protein (cell surface–localized).*

3.5.3 SDS-PAGE [13]

1. Prepare 0.75-mm thick polyacrylamide separating and stacking gels. *The acrylamide percentage depends on the molecular weight of the studied protein and should position the protein just below the middle of the gel to allow maximal separation of different forms of the protein.*
2. Load 8 μL of each sample (and 3 μL of the total lysates on a separate gel to check labeling efficiency). Not using the outer lanes of the gel, load 1× sample buffer in the outside as well as empty lanes to prevent "smiling" of the bands. *When reduced and non-reduced samples are loaded in adjacent lanes in SDS-PAGE, DTT can diffuse into the non-reduced lanes. Either one or two lanes should be left open between the two samples, or an excess quencher should be added to all samples (including the non-reduced ones) just before loading. We frequently use 100 mM* N*-ethylmaleimide (final concentration in the sample) for this purpose.*
3. Run each gel at max speed at constant current (25 mA per gel for Hoefer minigels) until the dye front is at the bottom of the gel. *Maximum speed without overheating limits lateral diffusion and guarantees sharper bands.*
4. Stain the gel with Coomassie brilliant blue for 5 min and destain to visualize antibody bands.
5. Neutralize gels for 1–3 × 5 min. *If incubated too long in the absence of acid, bands will become diffuse. A minimum time of neutralization is needed to prevent precipitation of salicylic acid, if gels are too acidic.*
6. Treat gels with enhancer solution for 15 min. *Do not incubate longer than 15 min to prevent non-specific signal.*
7. Dry gels at 80 °C on 0.4-mm Schleicher & Schuell filter paper.
8. Expose to film at −80 °C or to a phosphor-imaging screen.

Solutions for SDS-PAGE

Coomassie stain: 0.25% (w/v) Coomassie brilliant blue in destain

Destain: 30% (v/v) methanol and 10% (v/v) acetic acid in H_2O

Neutralizer: 30% (v/v) methanol in PBS

Enhancer: 1.5 M sodium salicylate in 30% (v/v) methanol in H_2O

Various types of native gels can be used to study the conformation of proteins, oligomerization, aggregation, and the interactions between different proteins. The mobility depends on both the protein's charge and its hydrodynamic size. Blue native gels [86] are often are used for membrane proteins.

Two-dimensional SDS-PAGE

1. Load samples on a tube gel and run, or run on a slab gel with pre-stained markers on either side of the lane. *The first dimension can be a native gel or a non-reducing SDS-PA gel, an IEF gel, or virtually any other kind.*
2. Extract tube gel from capillary, or cut the lane of interest out of the first dimension slab gel.
3. Boil the tube or strip for 10 min in reducing sample buffer.
4. Place the tube or strip on an SDS-polyacrylamide slab gel.
5. A reduced sample can be run in a separate lane close to the edge of the slab gel.
6. Run in the second dimension as a regular slab gel.

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