POST-TRANSLATIONAL MODIFICATION: SPECIALIZED APPLICATIONS

14

continued

CHAPTER 14
Post-Translational Modification: Specialized Applications

INTRODUCTION

The amino acid sequence of a protein determines its structural and functional properties. Yet the primary structure of a polypeptide is not a complete description of its chemical constituents; modifications of the polypeptide backbone may alter the intracellular destination, as well as modify functional properties of the polypeptide in question. Some of these modifications occur as the polypeptide rolls off the ribosome; others take place on complete polypeptide chains; or both co- and post-translational modification may occur on the same polypeptide, as exemplified by protein-bound carbohydrates.

A number of post-translational modifications may be inferred from the primary structure or from the open reading frame established by DNA sequencing. These include potential cleavage sites for signal peptidase in the case of secretory or type-I membrane proteins; cleavage sites for other proteases that process inactive precursors into active polypeptides, as exemplified by neuropeptide precursors; consensus sequences for N-linked glycosylation; and C-terminal modification by isoprenoid moieties in conjunction with carboxymethylation. Similarly, consensus sequences for certain types of phosphorylation events have been established. Although for many of these modifications consensus sequences indeed indicate their possible occurrence, it is not always safe to conclude that such modifications therefore necessarily take place. For that reason, methods to identify and characterize these modifications are essential.

All of the naturally occurring amino acids can occur in proteins in a modified form. In this manual, separate chapters are devoted to the most intensively and possibly widespread protein modifications, glycosylation (Chapter 12) and phosphorylation (Chapter 13). In this chapter, methods for the study of other protein modifications will be addressed.

The presence of disulfide bonds stabilizes the structure of proteins and allows them to survive in the extracellular milieu. This type of modification cannot be inferred from primary structure without the aid of homologous proteins for which the disulfide bonding pattern has been established by chemical analysis, as described elsewhere in this manual (*UNIT 7.3*). Furthermore, in guiding protein folding and in allowing the formation of higher-order structures such as homo- or hetero-oligomers, disulfide bond formation is an important aspect of how the conformation of a mature, functional protein is reached. *UNIT 14.1* describes the technology with which to study disulfide bond formation in living cells and in a completely cell-free system.

Modification of proteins by lipid moieties allows proteins to interact with membranes. The functional properties of a number of proteins depend critically on this modification, and establishing whether or not acylation (*UNIT 14.2*) or isoprenylation (*UNIT 14.3*) have occurred is therefore important. There are other modifications that occur at distinct subcellular localizations, such as the modification of tyrosine with sulfate.

Contributed by Hidde L. Ploegh and Ben M. Dunn
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The effect of oxygen on proteins can lead to modifications of cysteine, tyrosine, aspartic acid, and asparagine, as well as producing new reactive carbonyl groups. Methods for quantifying the products of such oxidative damage are given in *UNIT 14.4*.

Future supplements will describe additional post-translational modifications to aid the investigator in achieving a more complete description of the protein under study.

Hidde L. Ploegh and Ben M. Dunn

Analysis of Disulfide Bond Formation

Most proteins synthesized in the endoplasmic reticulum (ER) in eukaryotic cells and in the periplasmic space in prokaryotes are stabilized by disulfide bonds. Disulfide bonds are of two types: intrachain (within a polypeptide chain) and interchain (between separate chains). Intrachain disulfide bonds are formed during cotranslational and post-translational folding of a newly synthesized protein. Most interchain disulfide bonds are formed at a later stage in the maturation process and establish covalent links between subunits in oligomeric proteins.

Disulfide bond formation can be followed in cultures of intact cells (Basic Protocol 1 and Alternate Protocols 1 and 2) or in an in vitro translation system containing isolated microsomes (Basic Protocol 2 and Alternate Protocol 3). First, the newly synthesized protein of interest is biosynthetically labeled with radioactive amino acids in a short pulse. The labeled protein is chased with unlabeled amino acids. At different times during the chase, a sample is collected, membranes are lysed with detergent, and the protein is isolated by immunoprecipitation (Support Protocol 1). The immunoprecipitates are analyzed for the presence of disulfide bonds by SDS-PAGE with and without prior reduction (Support Protocol 2). The difference in mobility observed between the gels with unreduced and reduced samples is due to disulfide bonds in the unreduced protein.

CAUTION: Radioactive materials require special handling. See *APPENDIX 2B* concerning safe use of radioisotopes.

ANALYSIS OF DISULFIDE BOND FORMATION IN INTACT MONOLAYER CELLS

BASIC PROTOCOL 1

In vivo, disulfide bond formation can be examined in cells growing in monolayers on tissue culture dishes (Braakman et al., 1991). This allows multiple wash steps in a short period of time and makes it easy to use short pulse and chase times. In addition, adherent cells are suitable for studies requiring frequent changes of medium with very different composition. When cells do not adhere or when low volumes of medium are desirable (e.g., when expensive additives are needed), the analysis can be done in suspension (see Alternate Protocol 1). This method detects cotranslational and post-translational disulfide bond formation; it is also possible to analyze post-translational disulfide bond formation (see Alternate Protocol 2).

Materials

Adherent cells
Tissue culture medium containing methionine, 37°C
Wash buffer (see recipe), 37°C
Depletion medium (see recipe), 37°C
Labeling medium (containing 125 to 250 μCi/ml [^{35}S]methionine; see recipe), 37°C
Chase medium (see recipe), 37°C
Stop buffer (see recipe), 0°C
Lysis buffer (see recipe), 0°C

60-mm tissue culture dishes, sterile
37°C humidified 5% CO_2 incubator
37°C water bath with rack or insert to hold tissue culture dishes (e.g., Unwire racks for 15- and 50-ml tubes, Nalge)
Aspiration flask for radioactive waste
Flat, wide ice pan with fitted metal plate (e.g., VWR Scientific)
Cell scraper

Additional reagents and equipment for immunoprecipitation (see Support Protocol 1) and nonreducing and reducing SDS-PAGE (see Support Protocol 2)

NOTE: The volumes described here are for a 60-mm dish of cells. Volumes must be adjusted, based on the surface area of the dish, for other sizes.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Perform pulse and chase

1. Set up cultures of adherent cells in tissue culture medium containing methionine in 60-mm tissue culture dishes so the cells will form a subconfluent monolayer on the day of the experiment. Incubate at 37°C.

   The experiment requires at least 1 dish per time point, and each dish should contain ≥10^6 cells.

2. Set up 37°C water bath with rack or insert for tissue culture dishes. Check that the water level is in contact with the bottom of a tissue culture dish but does not allow it to float if the lid is removed. Arrange the aspiration flask for radioactive waste so the pipet easily reaches dishes in the water bath.

3. Rinse cells twice with 2 ml wash buffer. Aspirate wash buffer and add 2 to 2.5 ml depletion medium. Incubate 15 to 30 min at 37°C in an incubator.

   This step depletes methionine in the cultured cells.

4. Remove dishes from the incubator and place on rack in 37°C water bath.

5. Pulse-label the cells, one dish at a time: aspirate depletion medium, add 400 µl labeling medium containing 50 to 100 µCi [^{35}S]methionine, and incubate 1 to 2 min on rack in 37°C water bath.

   The pulse time should be equal to or shorter than the time required to synthesize the protein of interest (assuming a rate of 4 to 5 amino acid residues/sec) and long enough to detect the protein.

   If the protein of interest does not contain many methionines, or if it contains several cysteines, it may be worthwhile to deplete cysteine as well as methionine and label with [^{35}S]methionine + [^{35}S]cysteine (50 to 100 µC; the total ratio depends on the protein) or with the same quantity of unpurified [^{35}S]methionine, which usually contains ~15% (v/v) [^{35}S]cysteine as well. The stabilized form of unpurified methionine (e.g., [^{35}S]in vitro labeling mix, Amersham) is less volatile and should be used for short pulses in a water bath to minimize radioactive contamination of air, pipets, and equipment.

For 0-min chase interval

6a. Add 2 ml chase medium to stop the pulse at precisely the end of the labeling interval. Rock gently to mix.

7a. Aspirate chase medium as quickly as possible. Transfer dish to aluminum plate on ice pan. Immediately add 2.5 ml cold stop buffer to end the chase.

For all other chase intervals

6b. At precisely the end of the pulse labeling interval, add 2 ml chase medium to start the chase. Rock gently to mix. Aspirate medium and add 2 ml chase medium again. Incubate in a 37°C incubator or 37°C water bath for the desired chase intervals.

   Choice of chase times is determined by the time it takes a protein to fold and form disulfide bonds. Generally, the first chase interval is equal to pulse time, the next approximately double that, and so on (e.g., 2, 5, 10, 20, and 40 min).

For short chase intervals, dishes can be incubated in a water bath, but for chase intervals >15 to 20 min, pH is best maintained in an incubator.

7b. At the end of the chase interval, aspirate chase medium and transfer dish to aluminum plate on ice pan. Add 2.5 ml cold stop buffer to end the chase.

Cold stop buffer is used to stop all cellular processes. Cells may be left ≤30 min on ice in stop buffer.

Prepare the lysate

8. Remove stop buffer and add 2.5 ml cold stop buffer.

9. Aspirate dish as dry as possible. Add 600 µl cold lysis buffer.

No incubation with lysis buffer is necessary when the buffer contains an alkylating agent. If alkylating agent is omitted from the lysis buffer, incubate 10 min with 20 mM N-ethylmaleimide (NEM) or longer with 20 mM iodoacetamide or 20 mM iodoacetic acid (typically 15 to 45 min).

10. Scrape dish and mix cell lysate with cell scraper. Transfer the lysate to a labeled 1.5-ml microcentrifuge tube.

11. Microcentrifuge 5 min at 12,000 rpm, 0°C, to pellet nuclei. Transfer supernatant (postnuclear lysate) to a clean 1.5-ml microcentrifuge tube.

12. Immediately analyze postnuclear lysate by immunoprecipitation (see Support Protocol 1 and *UNIT 13.2*) and nonreducing and reducing SDS-PAGE (see Support Protocol 2) or freeze it rapidly in liquid nitrogen and store at –80°C.

Lysate with alkylating agent can be stored 1 to 2 hr on ice; lysate without alkylating agent should not be stored >5 min on ice.

ANALYSIS OF DISULFIDE BOND FORMATION IN CELLS IN SUSPENSION

ALTERNATE PROTOCOL 1

When cells do not adhere adequately to a solid support, or when it is desirable to use small volumes of reagents for analyses, cells in suspension may be used to analyze disulfide bond formation. One advantage to this approach is that samples at different chase intervals may be collected from a single tube of labeled cells. However, wash steps cannot be included during the pulse-chase incubations because the washes take too much time. Prior to starting the experiment it is necessary to determine (1) the minimum volume for incubating the cells (x µl), (2) the number of chase time points (y), and (3) the desired sample volume (z µl). Approximately 10^6 cells should be used for each time point.

Additional Materials *(also see Basic Protocol 1)*

Culture of suspension cells >1000
10 mCi/ml [^{35}S]methionine (>1000 Ci/mmol; Amersham)
2× lysis buffer (see recipe)
Concentrated chase medium (see recipe)

50-ml polystyrene tube with cap, sterile
Beckman GPR cell centrifuge or equivalent

Additional reagents and equipment for immunoprecipitation (see Support Protocol 1) and nonreducing SDS-PAGE (see Support Protocol 2)

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

NOTE: Keep cells in suspension during incubations by gently swirling the tube at regular intervals.

1. Transfer suspension cells for analysis to a sterile 50-ml polystyrene tube with cap.

   Use ~10^6 cells per data point.

   If it is necessary to minimize the volumes of reagents used for the experiment, adherent cells can be removed from the dish by treating them with 0.25% (w/v) trypsin/0.2% (w/v) EDTA and resuspending them in a low-calcium medium.

2. Centrifuge cells 4 min at 500 × *g* (1500 rpm), 20° to 37°C. Resuspend pellet in 2*y* ml depletion medium and centrifuge again. Resuspend in 2*y* ml depletion medium. Incubate 15 to 30 min at 37°C.

   Pelleting conditions may vary with cell type.

3. Centrifuge cells 4 min at 500 × *g*, 20° to 37°C. Resuspend cells in *x* µl depletion medium in appropriate tube and place in water bath.

4. At the start of pulse, add 50 to 100 µCi undiluted [^{35}S]methionine per time point to the cell suspension and mix by swirling. Incubate for the pulse period.

5. At the end of the pulse, add ≥4 vol (4 × *x* µl) concentrated chase medium. Mix by swirling.

   The total volume after addition of concentrated chase medium should be slightly more than y × z µl to allow for loss due to evaporation.

   The final concentrations of chase medium components must be identical to those for the chase medium used in Basic Protocol 1 (see recipe for chase medium). Calculate the concentrations needed for the concentrated chase medium. A 1.25× solution is appropriate if 4 vol concentrated chase medium is added to the labeling mixture; for other added volumes, the concentrations must be adjusted accordingly.

6. Immediately collect the first sample of volume *z*. Add an equal volume of 2× lysis buffer, mix well, and place on ice.

7. At every chase time point, take a sample, add an equal volume of 2× lysis buffer, mix well, and place on ice.

   After the sample for the last chase time point is collected, the tube of cells should be almost empty.

8. Immediately analyze samples by immunoprecipitation (see Support Protocol 1) and nonreducing and reducing SDS-PAGE (see Support Protocol 2) or freeze rapidly in liquid nitrogen and store at –80°C.

ALTERNATE PROTOCOL 2

ANALYSIS OF POST-TRANSLATIONAL DISULFIDE BOND FORMATION IN INTACT CELLS

To determine the effect of certain conditions, such as ATP depletion, on folding, it may be necessary to separate the folding process from translation. This can be done by delaying folding until translation, glycosylation, and signal-peptide cleavage are complete. Folding of a disulfide-bonded protein may be prevented by incubating cells in reducing agent. Newly synthesized proteins cannot form disulfide bonds and often cannot fold when oxidation is prevented. When the reducing agent is removed after pulse-labeling, formation of disulfide bonds and folding may proceed (Braakman et al., 1992a,b; see Fig. 14.1.1).

Wash the cells and deplete the methionine (see Basic Protocol 1, steps 2 and 3). Add dithiothreitol (DTT; *APPENDIX 3A*) to labeling medium to give a final concentration of 5 mM. Perform a pulse-chase experiment and prepare lysates (see Basic Protocol 1, steps 4 to 11).

It may be necessary to optimize the final concentration of DTT in labeling medium for each cell type. During long incubations, DTT may affect cellular ATP levels. Efficiency of incorporation may also be slightly diminished by the presence of DTT. The duration of pulse labeling may be increased to improve incorporation of label in protein.

Before the effects of different conditions on folding are tested, the efficiency, rate, and outcome of post-translational folding should be compared to those of cotranslational folding.

ANALYSIS OF DISULFIDE BOND FORMATION IN ROUGH ENDOPLASMIC RETICULUM–DERIVED MICROSOMES

BASIC PROTOCOL 2

Formation of disulfide bonds in proteins which possess signal sequences that target the protein to the endoplasmic reticulum can also be studied with an in vitro translation/rough endoplasmic reticulum–derived microsome translocation and folding system (Fig. 14.1.1). ^{35}S-labeled proteins are generated by the translation of mRNA with a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Translation is carried out in the presence of dog pancreas microsomes; this combination permits cotranslational insertion of ^{35}S-labeled protein into microsomes. An oxidizing agent (i.e., oxidized glutathione, GSSG; *APPENDIX 3A*) is present during translation to allow monitoring of cotranslational or vectorial oxidation from the N- to the C-terminus of the protein, the mechanism by which proteins acquire their disulfide bonds under normal physiological conditions.

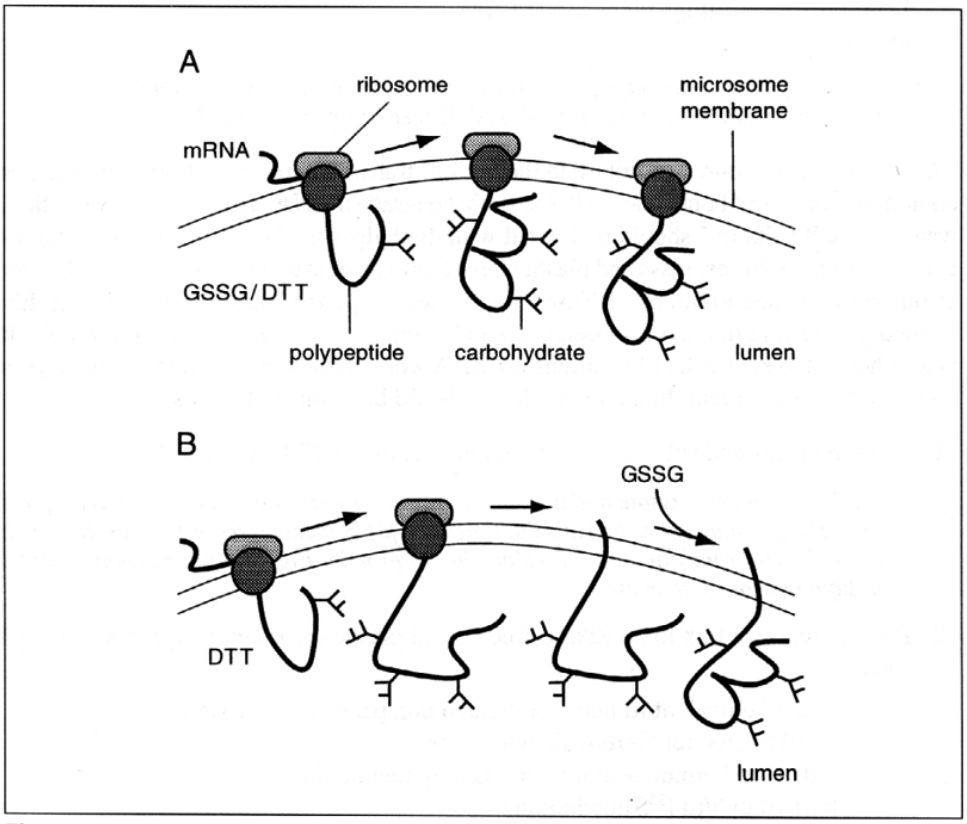

Figure 14.1.1 In vitro translation/rough endoplasmic reticulum–derived microsome translocation and folding system. (**A**) For cotranslational folding, the protein is translocated into microsomes containing an oxidizing environment (GSSG). This provides an opportunity for the protein to fold vectorially during the translation and translocation processes. (**B**) For post-translational folding, oxidizing agent is added after translation and translocation, permitting synchronization and isolation of the folding process.

Oxidation of proteins in microsomes provides a system where the experimental conditions can be more extensively controlled and manipulated. The oxidation state of the protein can be trapped by alkylation with *N*-ethylmaleimide (NEM) and monitored by immunoprecipitation (see Support Protocol 1) and nonreducing and reducing SDS-PAGE (see Support Protocol 2).

Materials

1 equivalent/µl nuclease-treated dog pancreas microsomes (Promega)
Rabbit reticulocyte lysate treated with ATP-regenerating system and nucleases (Promega)
1 mM amino acid mixture lacking methionine (Promega)
10 mCi/ml [^{35}S]methionine (1000 Ci/mmol, Amersham)
100 mM dithiothreitol (DTT)
RNase-free H_2O (e.g., DEPC-treated; see recipe)
23 U/ml RNase inhibitor (e.g., RNasin, Promega)
1 µg/µl mRNA for the protein of interest
100 mM oxidized glutathione (GSSG; *APPENDIX 3A*), titrated to neutrality with KOH
120 mM *N*-ethylmaleimide (NEM) in 100% ethanol (prepare from 1 M stock; see recipe)
Lysis buffer (see recipe)
2× SDS sample buffer (*UNIT 10.1*; optional)

27°C water bath
1.5-ml microcentrifuge tubes, RNase-free
Ice bath

Additional reagents and equipment for immunoprecipitation (see Support Protocol 1) and nonreducing SDS-PAGE (see Support Protocol 2)

NOTE: The major source of failure in the in vitro translation/translocation of proteins is contamination with ribonucleases (RNases) that degrade mRNA. To avoid contamination, water and salt solutions should be treated with diethylpyrocarbonate (DEPC) to chemically inactivate RNases; glass and plasticware should be treated with DEPC-treated water or otherwise treated to remove RNase activity (see recipe for DEPC treatment). Freshly opened plasticware that has not been touched by unprotected hands is also acceptable. It is also helpful to keep a set of solutions for RNA work alone to ensure that "dirty" pipets do not contaminate them. In addition, gloves should be worn at all times.

1. Thaw reagents and solutions rapidly with agitation in 27°C water bath.

   Rabbit reticulocyte lysate and dog pancreas microsomes should be stored in aliquots of working volumes (i.e., 6 µl and multiples of 6 µl). Repeated freeze-thaw cycles result in inefficient translation and translocation of protein. Freeze other reagents rapidly in liquid nitrogen after use.

2. Prepare reaction mix in an RNase-free 1.5-ml plastic microcentrifuge tube (96.7 µl total):

   6 µl 1 equivalent/µl nuclease-treated dog pancreas microsomes
   52 µl treated rabbit reticulocyte lysate
   2 µl 1 mM amino acid mixture lacking methionine
   8 µl 10 µCi/µl [^{35}S]methionine
   1 µl 100 mM DTT
   16 µl RNase-free H_2O
   4 µl 23 U/µl RNase inhibitor
   4 µl 1µg/µl mRNA for the protein of interest
   3.7 µl 100 mM GSSG.

This reaction mix is enough for 10 samples. Adjust the volumes proportionately for more or fewer samples. Each sample represents a different time point.

mRNA for the protein of interest can be transcribed from cDNA for the protein of interest into a commercially available vector (e.g., pBluescript, Stratagene) which contains both T7 and T3 promoters for RNA polymerases.

3. Mix thoroughly with a pipet. Incubate at 27°C.

   Set pipettor to less than the total volume of reaction mixture to avoid introducing air bubbles. Mix by repeatedly taking the mixture up and gently expelling it with the pipettor.

4. At the appropriate time (e.g., 0.5, 1, 1.5, 2, 3,...*n* hr), transfer 9-µl samples to separate tubes and add 2.4 µl of 120 mM NEM in ethanol (25 mM final) to alkylate proteins. Incubate 10 min on ice.

   In vitro translation of proteins is 3- to 5-fold slower than in vivo translation, so extended time points are required.

5. Add 600 µl lysis buffer to each alkylated sample *or* add 30 µl of 2× sample buffer for direct analysis of total proteins translated.

6. Analyze lysate by immunoprecipitation (see Support Protocol 1) and nonreducing SDS-PAGE (see Support Protocol 2).

ANALYSIS OF POST-TRANSLATIONAL DISULFIDE BOND FORMATION IN ROUGH ENDOPLASMIC RETICULUM–DERIVED MICROSOMES

ALTERNATE PROTOCOL 3

Post-translational oxidation is performed by translating the protein under reducing conditions in the presence of microsomes to allow accumulation of translocated and glycosylated proteins with the signal sequence removed and lacking disulfide bonds. Oxidizing agents are added post-translationally to initiate oxidation (see Fig. 14.1.1).

Perform in vitro translation and analysis as for the previous method (see Basic Protocol 2) with the following exceptions in the indicated steps.

2. Omit oxidized glutathione (GSSG) from the reaction mixture (to make 93 µl).

3. After the initial incubation of the reaction, add 4.2 µl of 100 mM GSSG and incubate 1 hr more at 27°C before proceeding to alkylate and analyze proteins (steps 4 to 8).

IMMUNOPRECIPITATION OF LYSATES

SUPPORT PROTOCOL 1

The protein of interest is precipitated from lysates of cells or microsomes using a specific antibody bound to *Staphylococcus aureus* cells or Protein A–Sepharose beads (beads give less background but are twice as expensive as the cells). The immunoprecipitate is then analyzed by nonreducing and reducing SDS-PAGE (see Support Protocol 2).

Materials

10% (w/v) killed, fixed *Staphylococcus aureus* cells (Zymed)
Antibody against protein of interest
Lysate from pulse-chase labeled cells or microsomes (see Basic Protocol 1 or 2 or Alternate Protocol 1, 2, or 3)
Immunoprecipitation wash buffer (see recipe), 37°C
TE buffer, pH 6.8 (see recipe)
2× nonreducing sample buffer (see recipe)
200 mM dithiothreitol (DTT)
Rotator

Additional reagents and equipment for nonreducing and reducing SDS-PAGE (see Support Protocol 2)

1. Add 60 µl of 10% killed, fixed *Staphylococcus aureus* cells to 0.5 to 100 µl antibody. Rotate 1 hr at 4°C.

   The amount of antibody used depends on the antibody and its concentration.

   The time required to bind antibody to S. aureus cells may vary from 20 min to several hours.

   Alternatively, 60 µl of 10% (w/v) Protein A–Sepharose beads, washed five times in an appropriate buffer, may be used instead of S. aureus cells.

2. Add 100 to 600 µl postnuclear lysate. Incubate 1 hr at 4°C, with shaking.

   If background is high, preincubate the lysate with S. aureus cells without antibody 1 hr at 4°C. Microcentrifuge the cells 4 min at 2000 × g, room temperature, and transfer the cleared lysate to antibody-coated S. aureus cells.

3. Pellet cells by microcentrifuging 4 min at 2000 × *g* (5000 rpm), room temperature. Remove supernatant and resuspend cells in 1 ml wash buffer. Shake 5 min. Repeat wash once and pellet cells.

   The temperature of the wash can affect the level of background; generally, the higher the temperature, the lower the background. Typical wash temperatures range from 10°C to room temperature.

   Protein A–Sepharose beads can be pelleted by centrifuging 1 min at 8000 × g (10,000 rpm), room temperature.

4. Aspirate the supernatant. Add 20 µl TE buffer. Shake 5 min or until cells are resuspended.

5. Add 20 µl of 2× nonreducing sample buffer without reducing agent and vortex. Boil 5 min at 95°C and vortex.

6. Pellet *S. aureus* cells by microcentrifuging 4 min at 12,000 × *g* (12,000 rpm) to give nonreduced sample in the supernatant.

7. Transfer 20 µl supernatant to a tube containing 2 µl of 200 mM DTT and vortex. Boil 5 min at 95°C. Microcentrifuge briefly at 12,000 × *g* to give reduced sample.

8. Analyze nonreduced and reduced samples by SDS-PAGE (see Support Protocol 2).

   Samples can be rapidly frozen in liquid nitrogen and stored at –80°C, but storage may increase background.

SUPPORT PROTOCOL 2

NONREDUCING AND REDUCING SDS-PAGE

Immunoprecipitates (see Support Protocol 1) are analyzed by nonreducing SDS-PAGE to detect changes in mobility due to disulfide bond formation.

Materials

Samples in 1× sample buffer (see Support Protocol 1)
2× nonreducing sample buffer (see recipe)
PBS (*APPENDIX 2E*)/30% (v/v) methanol
1.5 M salicylate/30% (v/v) methanol

pH paper
Whatman 3MM filter paper

Additional reagents and equipment for SDS-PAGE minigel with Laemmli buffers (*UNIT 10.1*) and Coomassie blue staining and destaining (*UNIT 10.5*)

1. Prepare a 1- or 0.75-mm thick polyacrylamide separating and stacking minigel.

   Percent acrylamide depends on molecular weight of the protein.

2. Load 8 µl of each sample. Load 1× nonreducing sample buffer in the two lanes next to the samples.

   When nonreduced and reduced samples are to be loaded on the same gel, leave two empty lanes between the two sample types, and load empty lanes with 1× nonreducing sample buffer. If nonreduced and reduced samples need to be loaded next to each other, cool the samples and add NEM to a final concentration of 100 mM to both sets of samples. Mix and pellet the cells before loading the samples on the gel.

3. Run gel ~1 hr at 20 to 25 mA until the dye front is close to or at the bottom of the gel.

4. Stain the gel, including the stacking gel, with Coomassie blue stain and destain (see *UNIT 10.5*).

   Stacking gels often contain unreduced or aggregated protein.

5. Neutralize gel by incubating it in three changes of PBS/30% methanol, 5 min each, until pH >6. Check the pH with pH paper.

6. Treat gel 30 min in 1.5 M salicylate to enhance.

   Salicylate is used as a safer, less expensive, and faster way to enhance the signal 3- to 5-fold. Fluorography can also be enhanced using commercial solutions (e.g., Enhance, DuPont or Amplify, Amersham).

7. Dry gel onto Whatman 3MM filter paper. Autoradiograph/fluorograph at –80°C.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2E****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Chase medium

Complete tissue culture medium appropriate for the cells containing:
20 mM HEPES (sodium salt), pH 7.3 (see recipe)
5 mM methionine (see recipe)
1 mM cycloheximide (optional; see recipe)
Prepare fresh daily

Add HEPES, methionine, and optional cycloheximide from concentrated stocks (see recipes).

When labeling is done with cysteine, the chase medium should also contain 5 mM cysteine (from 1 M stock; see recipe).

The chase medium may contain fetal bovine serum or another protein source, especially when chase times are long. Protein should be omitted when more defined conditions are needed. When the reducing agent dithiothreitol (DTT) is added, proteins present in the medium will partly quench the reductant.

Concentrated chase medium

Adjust the concentration of chase medium components (see recipe) so the final concentrations are 20 mM HEPES, 5 mM methionine, and 5 mM cycloheximide after the concentrated chase medium has been added to the labeling medium for cells in suspension (see Alternate Protocol 1).

Cycloheximide, 500 mM stock

28.1 g cycloheximide
H_2O to 100 ml
Store 1- to 2-ml aliquots ≤2 years at –20°C

Thaw and warm to room temperature to completely dissolve before using. Do not freeze and thaw more than three times.

Cysteine, 1 M stock

12.12 g cysteine
H_2O to 100 ml
Store 1- to 2-ml aliquots ≤2 years at –20°C

Thaw and warm to room temperature to completely dissolve before using. Do not freeze and thaw more than three times.

Depletion medium

Methionine-free tissue culture medium containing:
0.026 M sodium bicarbonate (2.2 g/liter)
20 mM HEPES, pH 7.3 (from 1 M stock; see recipe)
Store ≤1 year at 4°C

Diethylpyrocarbonate (DEPC) treatment of solutions and labware

CAUTION: Wear gloves and use a fume hood when working with DEPC because it is a suspected carcinogen.

Solutions: Add 0.2 ml DEPC per 100 ml of the solution to be treated. Shake vigorously to dissolve DEPC. Autoclave the solution to inactivate remaining DEPC.

Any water or salt solutions used in RNA preparation should be treated with DEPC. Note that solutions containing Tris cannot be effectively treated with DEPC because Tris reacts with DEPC to inactivate it.

Labware: Rinse glass and plasticware thoroughly with DEPC solution. Alternatively, bake glassware 4 hr at 300°C; rinse plasticware with chloroform, or use fresh plasticware straight from a package that has not been touched by unprotected hands. Wear gloves for all manipulations.

Note that autoclaving alone will not fully inactivate many RNases.

Ethylenediaminetetraacetic acid (EDTA), 200 mM stock

18.61 g EDTA
180 ml H_2O
10 mM NaOH added dropwise with mixing just until EDTA dissolves
Adjust pH to 7.3
H_2O to 250 ml
Store at 4°C

For EDTA, pH 6.8, adjust the pH to 6.8.

HEPES (N-2-hydroxyethylpiperidine-N′-ethanesulfonic acid), 1 M stock

119.15 g HEPES (sodium salt)
400 ml H_2O
Adjust pH to 7.3 with 10 M NaOH
H_2O to 500 ml
Store 100-ml aliquots at –20°C and thawed aliquots at 4°C

Immunoprecipitation wash buffer

PBS (*APPENDIX 2E*)/0.5% (v/v) Triton X-100 *or*
PBS/150 mM NaCl
Store at 4°C or room temperature

For every protein-antibody combination, the optimal wash buffer needs to be determined. The two buffers listed are particularly mild wash buffers that will maintain most antigen-antibody interactions but may lead to high background.

To decrease background, other detergents or multiple detergents may be added, salt concentration may be increased, or a combination of the two may be used. SDS at a concentration ≥0.05% (w/v) may be especially helpful, with or without the quenching effect of added nondenaturing detergents.

Labeling medium

Methionine-free tissue culture medium containing:
125 to 250 µCi [^{35}S]methionine/ml
Prepare fresh for each experiment (400 µl per chase time point)

Addition of 50 to 100 µCi in 400 µl to a 60-mm-diameter dish (~3–5 × 10^6 cells) should be sufficient to visualize a 1- to 2-min-pulse-labeled protein that is expressed to a high level in the cell.

[^{35}S]methionine + [^{35}S]cysteine or unpurified [^{35}S]methionine + [^{35}S]cysteine can be used to label proteins that contain few methionines but several cysteines.

Lysis buffer

PBS (APPENDIX 2E) or similar buffer containing:
0.5% (v/v) Triton X-100
1 mM EDTA, pH 6.8 (see recipe), added just before use
20 mM NEM (see recipe), added just before use
1 mM PMSF (see recipe), added just before use
Small peptide mixture (10 µg/ml each final; see recipe), added just before use
Use within 3 hr

Add EDTA, NEM, and protease inhibitors (PMSF and small peptide mixture) from concentrated stocks (see recipes).

For 2× lysis buffer, double the concentrations of reagents.

Depending on the protein analyzed and on the purpose of the experiment, a variety of detergents may be used. Triton X-100 may disrupt noncovalent interactions between proteins. Milder detergents that can be used include CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), deoxycholate, octylglucoside, or a mixture of detergent and lipid.

Methionine, 200 mM stock

2.984 g methionine
H_2O to 100 ml
Store 1- to 2-ml aliquots ≤2 years at –20°C

Thaw and warm to room temperature to completely dissolve before using. Do not freeze and thaw more than three times.

N-ethylmaleimide (NEM), 1 M stock

12.5 g NEM
100 ml 100% ethanol
Store 1- to 2-ml aliquots ≤2 years at –20°C protected from light

NEM is sensitive to light and to hydrolysis in water. If it is frozen and thawed too many times or exposed to too much light, the solution will turn yellow and the NEM will precipitate.

Nonreducing sample buffer, 2×

400 mM Tris·Cl, pH 6.8
6% (w/v) SDS
20% (v/v) glycerol
2 mM EDTA, pH 6.8 (from 200 mM stock; see recipe)
0.01% (w/v) bromphenol blue

Phenylmethylsulfonyl fluoride (PMSF), 200 mM stock

3.484 g PMSF
10 ml anhydrous isopropanol
Store in 500-µl aliquots ≤2 years at –20°C

PMSF is highly unstable in water (the half life is ~30 min at 37°C and a few hours on ice). Add this stock to solution immediately before use.

Small peptide mixture stock

10 mg/ml chymostatin in dimethyl sulfoxide (DMSO)
10 mg/ml leupeptin in DMSO
10 mg/ml antipain in DMSO
10 mg/ml pepstatin in DMSO
Store in 10-µl aliquots ≤2 years at –20°C
Use at a final concentration of 10 µg/ml each

Stop buffer

Wash buffer (see recipe) containing salts only
20 mM NEM (from 1 M stock; see recipe)
Prepare fresh and store on ice <5 hr

NEM may be replaced by iodoacetamide or iodoacetic acid, which react more slowly with free –SH groups. They are less specific than NEM and penetrate cells more slowly than NEM, but they irreversibly carboxymethylate the –SH group, whereas NEM may dissociate slowly. Deblocking of –SH groups after NEM treatment is not a problem if cells are stored ≤2 hr on ice.

TE buffer, pH 6.8

10 mM Tris·Cl, pH 6.8
1 mM EDTA, pH 6.8 (from 200 mM stock; see recipe)
Store at 4°C

Wash buffer

PBS (*APPENDIX 2E*) containing 0.9 mM Ca^{2+}/0.5 mM Mg^{2+}, Earles balanced salt solution, or other isotonic buffer containing salts only.

For most cells, calcium ions are crucial to maintain adherence to the plastic dishes, especially to withstand many washes. When cost is not an issue, all washes can be performed using depletion medium. However, depletion medium may not be used as a substitute in stop buffer.

COMMENTARY

Background Information

Folding of a newly synthesized protein can be followed by assaying for conformational changes in the molecule immediately after synthesis. For this type of analysis, a large number of antibodies that recognize specific epitopes are required to analyze the changes in the complete molecule. In a mature protein that is stabilized by intrachain disulfide bonds (which is true of most proteins synthesized in the endoplasmic reticulum), the formation of these cross-links is indicative of folding (Creighton, 1986). A disulfide bond can be formed only when the conformation allows the two participating cysteines to be in close proximity. With only few antibodies available, the rate and extent of folding of a protein can be examined grossly through detection of disulfide bond

formation. Of course, disulfide bond formation is not identical to folding; therefore, a comparison of disulfide bond formation and conformational changes is required for every protein studied.

When a disulfide bond–containing protein is prepared for SDS-PAGE, it is routinely reduced. In nonreducing denaturing polyacrylamide gel electrophoresis, however, the protein is denatured with SDS, but the disulfide bonds remain intact. The consequence is a more compact conformation of the protein, resulting in a higher electrophoretic mobility; however, in some cases the protein may bind less SDS, resulting in a lower electrophoretic mobility. Most proteins will run faster in oxidized form than in reduced form.

The choice of radioactive labeling is dictated by the desire to follow a protein from the moment of synthesis. No other method permits following the life of a protein in a cell in such detail. Any other method of detection would require bulk production of proteins, which would be very difficult and nonphysiological.

The in vitro translation system coupled with dog pancreas microsomes has been traditionally employed to study the translocation of secretory, lysosomal, and many integral membrane proteins across the membrane of the endoplasmic reticulum (Blobel and Dobberstein, 1975). However, more recently it has been shown that microsomes possess a complete set of foldases and chaperones and thus provide an environment in which proteins can be oxidized rapidly and efficiently (Scheele and Jacoby, 1982; Marquardt et al., 1993; Hebert et al., 1995). Microsomes provide a system that can be controlled and manipulated to a much higher degree than intact cells. Direct access is provided to the lysate (equivalent to the cytosol in the cell), and intralumenal components can be manipulated with ionophores, detergents, toxins, or alkaline pH (Bulleid and Freedman, 1988; Nicchitta and Blobel, 1993; Hebert et al., 1995). Post-translational oxidation of proteins (see Alternate Protocol 2 and Alternate Protocol 3) can be used in both intact cells and microsomes to isolate the oxidation process from other biosynthetic events. Together, analysis of cellular and microsomal folding can be employed to dissect the folding pathway of a protein.

Critical Parameters

Wash buffer for adherent cells should contain Ca^{2+} and Mg^{2+} to maintain adherence to the plate, especially when there are several washes. If cost is not an issue, cells can be washed in depletion medium.

Labeling medium should contain 50 to 100 μCi ^{35}S-labeled methionine for ~3–5 × 10^6 cells. If the protein of interest has few methionines, or if it contains several cysteines, a combination of [^{35}S]methionine and [^{35}S]cysteine, or unpurified labeled methionine and cysteine, may be used for labeling. When short pulses are performed in a water bath, the stabilized version of unpurified methionine (^{35}S in vitro labeling mix, Amersham) should be used to minimize contamination of air, pipets, and incubators.

Cycloheximide in the chase medium will stop elongation of unfinished nascent peptide chains. When the kinetics of disulfide bond formation are being studied, cycloheximide should be added to the chase medium; however, it should be omitted when a maximum amount of incorporated label is required (Braakman et al., 1991).

An alkylating agent—e.g., *N*-ethylmaleimide, iodoacetamide, or iodoacetic acid—must be included in the stop buffer and lysis buffer to prevent artifactual formation of disulfide bonds. Initially, it is recommended to test at least two alkylating agents and compare the results with those obtained in the absence of alkylating agent. Ideally, there should be no difference in results obtained with the various alkylating agents, except possibly a change in electrophoretic mobility due to alkylation.

SDS-PAGE should be performed under nonreducing conditions. Reducing agents should be completely absent. If a reducing environment does exist in (one of) the samples, all samples should be quenched with at least a two-fold concentration of *N*-ethylmaleimide or another alkylating agent.

The translocation process is dependent upon free sulfhydryl groups, but oxidation requires an oxidizing environment. Thus, it would seem impossible for cotranslational oxidation to occur in the in vitro translation system with microsomes. However, it is possible to simultaneously create a reducing extralumenal compartment and an oxidizing intralumenal compartment, probably due to the presence of a putative oxidized glutathione (GSSH) transporter in microsomal membranes. The redox range over which these two processes occur efficiently is small. In addition, the redox potential is directly related to the pH, so pH must be carefully controlled.

Troubleshooting

The presence of disulfide bonds in a protein does not guarantee an electrophoretic mobility difference between reduced and oxidized forms, especially when a protein is large and disulfide bonds form small peptide loops. The largest shift can be expected at a late chase time when all disulfide bonds have been formed. If the presence of disulfide bonds is not certain and needs to be tested, the radioactive cell lysate can be treated with GSSG, followed by immunoprecipitation and analysis by nonreducing and reducing SDS-PAGE as described (see Support Protocol 1 and Support Protocol 2). Alternatively, free sulfhydryl groups can be labeled with [^{14}C]iodoacetamide before and after reduction.

When the mobility difference between reduced and oxidized protein is minimal, resolution might be improved by using a lower percentage acrylamide gel and/or changing the alkylating agent.

The treated reticulocyte lysate used here is optimized to efficiently translate a large range of mRNAs with an ATP-generating system, mixture of tRNAs, and salts. The concentration of salts greatly affects the translation efficiency. If the efficiency of translation is low, an untreated lysate should be used and optimized specifically for the mRNA for the protein of interest.

If there is difficulty in differentiating between translocated and untranslocated proteins in microsomes, untranslocated protein can be removed by protease digestion or centrifugation. Translocation of protein across microsomal membrane protects the protein from digestion by added proteases. Furthermore, dense rough microsomes can be pelleted easily by centrifugation, thereby isolating translocated proteins from extralumenal untranslocated proteins.

If the background is high in immunoprecipitation, try different immunoprecipitation wash buffers, using other detergents or multiple detergents, increased salt concentration, or a combination of both. SDS at concentrations ≥0.05% (w/v) may be especially helpful, with or without the quenching effect of added nondenaturing detergents.

Anticipated Results

In most cases, formation of disulfide bonds creates a more compact structure that results in an increase in the mobility of a protein in nonreducing SDS-PAGE (for representative results, see Braakman et al., 1992 a,b). The number of disulfide bonds and the distance between the cysteines in a sulfhydryl pair influences the observed change in mobility. During the chase, the protein will move from the more reduced position in the gel to the more oxidized position. For some proteins distinct oxidative intermediates may be observed, but for others, a fuzzy band may precede formation of native protein.

In reducing SDS-PAGE, ideally one band will be found in intact cells. Two bands will be found in the microsomal system—one for untranslocated protein and one for translocated protein. If the protein possesses a signal sequence (1.5 to 3.0 kDa) that is cleaved during translocation, a small mobility shift dependent upon the protein's molecular weight may be observed. However, differentiation of translocated and untranslocated proteins in reducing SDS-PAGE is best seen for multiglycosylated proteins, because each glycosylation step adds 2.5 kDa to the translocated protein. Modifications of oligosaccharides and other post-translational modifications can change the electrophoretic mobility as well, but these changes can be distinguished from disulfide bond formation by comparing reducing with nonreducing gels.

Time Considerations

Design and preparation of the experiment should take the most time. The very first time, this may take 1 or more days, depending on experience and available equipment. Preparation for routine experiments is around 1 hr. The pulse-chase portion requires 1 hr plus the maximum chase time. For rapidly folding proteins, folding can be over in 15 min; for others it may take hours. Cultures of cells should be started 1 or 2 days before the experiment so they will be subconfluent on the day of the experiment.

Pulse-chase, immunoprecipitation, and SDS-PAGE are optimally done in 1 day. It is possible to rapid-freeze lysates or immunoprecipitates in liquid nitrogen and store them at –80°C until further use, but freezing may lead to higher background and less reproducibility.

Literature Cited

Blobel, G. and Dobberstein, B. 1975. Transfer of proteins across membranes. II. Reconstitution of functional rough microsomes from heterologous components. *J. Cell Biol.* 67:852-862.

Braakman, I., Hoover-Litty, H., Wagner, K.R., and Helenius, A. 1991. Folding of influenza hemagglutinin in the endoplasmic reticulum. *J. Cell Biol.* 114:401-411.

Braakman, I., Helenius, J., and Helenius, A. 1992a. Manipulating disulfide bond formation and protein folding in the endoplasmic reticulum. *EMBO J.* 11:1717-1722.

Braakman, I., Helenius, J., and Helenius, A. 1992b. Role of ATP and disulphide bonds during protein folding in the endoplasmic reticulum. *Nature* 356:260-262.

Bulleid, N.J. and Freedman, R. 1988. Defective cotranslational formation of disulphide bonds in protein disulphide-isomerase-deficient microsomes. *Nature* 335:649-651.

Creighton, T.E. 1986. Disulfide bonds as probes of protein folding pathways. *Methods Enzymol.* 131:83-106.

Hebert, D.N., Foellmer, B., and Helenius, A. 1995. Glucose trimming and reglycosylation determine glycoprotein association with calnexin in the endoplasmic reticulum. *Cell* 81:425-433.

Marquardt, T., Hebert, D.N., and Helenius, A. 1993. Post-translational folding of Influenza hemagglutinin in isolated endoplasmic reticulum–derived microsomes. *J. Biol. Chem.* 268:19618-19625.

Nicchitta, C.V. and Blobel, G. 1993. Lumenal proteins of the mammalian endoplasmic reticulum are required to complete protein translocation. *Cell* 73:989-998.

Scheele, G. and Jacoby, R. 1982. Conformational changes associated with proteolytic processing of presecretory proteins allow glutathione-catalyzed formation of native disulfide bonds. *J. Biol. Chem.* 257:12277-12282.

Key References

Braakman et al., 1991. See above.

Describes the protocol in intact cells, results with influenza hemagglutinin, and considerations for ultra-short pulse times.

Hebert et al., 1995. See above.

Marquardt et al., 1993. See above.

Describes the protocol in microsomes.

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