The folding of influenza hemagglutinin was analyzed after in vitro translation and translocation into dog pancreas microsomes. Ectodomain folding of this membrane glycoprotein involves the formation of six intrachain disulfide bonds. After translation under reducing conditions, the folding process was initiated by the addition of oxidized glutathione or diamide. For correct folding a reduction-oxidation potential of −310 to −210 mV had to be reached in the bulk solution. At lower values, or after addition of other oxidants such as NAD or NADP, no HA disulfides formed. At more oxidizing values interchain disulfide-cross-linked aggregates were generated. Judging by their electrophoretic gel mobility and immunoreactivity, the folding intermediates observed in microsomes were indistinguishable from those previously seen in the endoplasmic reticulum of live cells. The kinetics of folding was also similar, but the efficiency being 43% was somewhat lower. The folding process was dependent on lumenal factors within the rough endoplasmic reticulum vesicles and also on some macromolecular component(s) present in the reticulocyte lysate. The results showed that dog pancreas microsomes provide a useful system for protein folding studies.

The in vitro refolding approach has provided most of the information that we have today about the principles of protein folding. A large body of refolding work has demonstrated that, under favorable conditions, denatured polypeptide chains can acquire an active native structure in the absence of additional folding factors (for recent reviews see Creighton, 1990; Gilbert, 1990; Jaenicke, 1991; Seckler and Jaenicke, 1992). It is, on the other hand, abundantly evident from recent studies in many cell systems that the conditions prevailing in the cell are not favorable for such spontaneous folding of newly synthesized proteins. Efficient folding of most proteins is, in fact, possible only because of the presence of chaperones, folding enzymes, metabolic energy, and a defined intracellular milieu (Freedman, 1989; Georgopoulos, 1992; Gething and Sambrook, 1992; Hartl et al., 1992; Hubbard and Sander, 1991; Rothman, 1989). In other words, although folding is determined by the amino acid sequence of the polypeptides, a diverse set of factors and conditions cooperate in making the folding process successful within the various compartments of the cell.

The endoplasmic reticulum (ER) is an organelle specializing in protein folding and oligomeric assembly (see Palade, 1975; Helenius et al., 1992). Secretory proteins, vacuolar proteins, and membrane glycoproteins are synthesized by membrane-bound ribosomes on the rough ER. Most are cotranslationally translocated into the ER lumen or inserted into the ER membrane. At least for larger proteins, the folding process begins already on the nascent chain and continues posttranslationally in the lumen (Bergman and Kuehl, 1979; Peters and Davidson, 1982). As a rule, the conformational maturation of proteins (including the oligomer formation) occurs in the ER or other pre-Golgi compartments, whereafter the proteins leave for the Golgi complex (Hurtley and Helenius, 1989; Klausner, 1989).

In keeping with its role as the most active folding compartment in the cell, the ER lumen is loaded with factors such as BiP/GRP78 (immunoglobulin heavy chain-binding protein, an hsp70 analogue) and protein disulfide isomerase (Bole et al., 1986; Haas and Wabl, 1983; Mazzarella and Green, 1987; Mazzarella et al., 1990; Noiva and Lennarz, 1992). Some of these luminal folding proteins are thought to be present in 0.1–1.0 mM concentrations (Hillson et al., 1984; Lyles and Gilbert, 1992a). In addition, the redox potential is maintained at a level more oxidizing than that in the cytosol (Hwang et al., 1992).

In our previous work, we have analyzed the folding of influenza hemagglutinin (HA) in living tissue culture cells. HA is a well characterized, 84-kDa type I membrane glycoprotein synthesized in the ER (Wiley and Skehel, 1987). By monitoring the formation of intrachain disulfide bonds and the appearance of antigenic epitopes, we have defined some of the steps during co- and post-translational folding of its ectodomain, and its homotrimer assembly (Braakman et al., 1992a, 1992b; Hurtley et al., 1989; Tatu et al., 1993).

To analyze in more detail the molecular events during HA folding, a simplified system is necessary. Ideally, such a system should retain the overall ER-like environment yet allow the milieu to be controlled and manipulated. To this end, we have here developed a cell-free translation approach using isolated rough ER-derived microsomes (RER microsomes). Taking advantage of some of our recent observations in intact

The abbreviations used are: ER, endoplasmic reticulum; DTT, dithiothreitol; HA, influenza hemagglutinin; NEM, N-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; RER, rough endoplasmic reticulum; MES, 4-morpholineethanesulfonic acid.
cells, we allowed HA translation and translocation to occur under reducing conditions, whereafter folding was induced post-translationally by the addition of oxidants. We showed that the microsomes supported efficient post-translational folding of HA and that the process was similar to that observed in living cells. The system allowed us to investigate some of the parameters that determine the efficiency and fidelity of folding.

**MATERIALS AND METHODS**

**Reagents**—The cell-free translation system (rabbit reticulocyte lysate, dog pancreas microsomes, amino acid mixture lacking methionine, dithiothreitol (DTT) and RNase) was purchased from Promega (Madinon, WI). In some experiments the dog pancreas microsomes were a gift from Dr. Reid Gilmore (University of Massachusetts Medical School, Worcester, MA). Trans 
\(^{35}\)S-label metabolic labeling reagent was obtained from ICN Biochemical Inc. (Irvine, CA). Oxidized glutathione (GSSG), which was stored frozen in stocks of 100 mM at pH 7.2, and reduced glutathione (GSH) were purchased from Fluka (Ronkonkoma, NY). Nikkol (C\(_{12}\)E\(_8\) or octaethyleneglycol dodecyl ether) was purchased from Calbiochem.

The pBluescript vector system was linearized beyond the 3'-end of the HA gene. The HA cDNA fragment was inserted into the pBluescript vector using the KpnI, T4 DNA polymerase, nucleotide triphosphates, and T7 RNA polymerase were from Boehringer Mannheim, Zyorbin (fixed and killed Staphylococcus aureus) was obtained from Zymed Laboratories Inc. (S. San Francisco, CA). All other chemicals employed including dialysis tubes, base, acid and deionized water were from Sigma.

The pBluescript vector system was linearized beyond the 3'-end of the cDNA using KpnI. T7 RNA polymerase was utilized to generate uncapped mRNA.

**Translation and Translocation of HA**—

\(^{35}\)S-Labeled HA was translated and translocated into dog pancreas microsomes (Scheele, 1983; Walter and Blobel, 1983) using the following lysis mixture: 52 \(\mu\)l of treated rabbit reticulocyte lysate, 2 \(\mu\)l of amino acid mixture without methionine, 8 \(\mu\)l of Trans 
\(^{35}\)S-label metabolic labeling reagent, 1 \(\mu\)l of 100 mM DTT, 16 \(\mu\)l of H\(_2\)O, 4 \(\mu\)l of RNase, 6 \(\mu\)l of dog pancreas microsomes, and 4 \(\mu\)l of HA mRNA. Post-transcription and translation were terminated after 1 h at 27 °C. Each reaction provided adequate labeled HA for 15 lanes on a 0.75 mm 7.5% SDS-PAGE mini-gel (Hoeffer, San Francisco, CA).

**Co- and Post-translational Folding of HA**—

For cotranslational folding, \(^{35}\)S-labeled HA was translated in the presence of dog pancreas microsomes as described except that 2 mM GSSG was added to the mixture. The concentration of GSSG used was experimentally determined so that disulfide bond formation in HA would occur but protein synthesis would not be inhibited. For the chase, cold amino acids were added. Samples were taken at different times of chase and folding stopped by alkylaition of free sulfhydryls with 20 mM N-ethylmaleimide (NEM, 1 \(\mu\)l of 120 mM NEM in 100% ethanol to 5 \(\mu\)l of sample lysate mixture) (Braakman et al., 1991; Creighton, 1978).

For post-translational folding studies, HA was synthesized and translocated as above except that no GSSG was added. The mixture thus contained DTT at the level of the reticulocyte lysate and 0.2 mM 2-mercaptopoethanol of the Trans 
\(^{35}\)S-label. Folding was initiated by the addition of the appropriate oxidant during chase and stopped as above by alkylaition.

The samples were either directly analyzed by SDS-PAGE or first solubilized in ice-cold 0.5% Triton X-100 in MNT buffer (20 mM MES, 100 mM NaCl, 20 mM Tris-HCl, pH 7.5) containing 20 mM NEM and protease inhibitors: 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 \(\mu\)g/ml each of chymostain, leupeptin, antipain, and pepstatin. For immunoprecipitation we employed 0.005% polyclonal rabbit aHA (unless otherwise indicated) and 3% Zysorbin. Routinely, 1 ml of the immunoprecipitation buffer was used per 5 \(\mu\)l of sample (one lane on a gel). The samples were mixed slowly by end-over-end spinning for 1 h at 4 °C. The immune-complexes were pelleted by centrifugation in an Eppendorf table top centrifuge at 2,500 \(\times\) g at 22 °C, and washed using 0.05% Triton X-100, 0.1% SDS, 0.3 M NaCl, 10 mM Tris-HCl, pH 8.6. The final pellets were resuspended in 30 \(\mu\)l of sample buffer, heated to 100 °C for 5 min, and centrifuged to remove the Zysorbin. The supernatants were analyzed by SDS-PAGE as described above.

**Reduction-oxidation Potentials**—Reduction-oxidation (redox) potentials were measured directly in the reaction mixtures with a MI-800-415 redox potential microelectrode (Microelectrodes Inc., Londonderry, NH) calibrated with pH 4.0 and 7.0 buffers saturated with quinhydrone with mV readings of 205 and 88, respectively, at 22 °C (as described in the redox electrode instruction brochure). NIKKOL Treatment of Microsomes—Nikkol treatment was employed to release soluble luminal proteins from the microsomes yet leave the microsomal membrane proteins membrane-associated (Connolly et al., 1988; Kelleher et al., 1992). RER microsomes were lysed with 0.05% Nikkol for 10 min at 0 °C. RER microsomes were concentrated by centrifugation through a sucrose cushion (50 mM triethanolamine, 1 mM DTT, 0.5 mM sucrose, pH 7.2) at 35,000 revolutions/min, 4 °C for 15 min in a Beckman TL-100 ultracentrifuge with a TL-55 rotor. Supernatant and pellet were both trichloroacetic acid precipitated. TCA precipitates were pelleted by centrifugation and resuspended in sample buffer for SDS-PAGE. SDS-polyacrylamide gels were either stained with Coomassie Blue or transferred to nitrocellulose with a Millipore MilliBlot-SDE system for immunoblotting with GRP94, ERp 72, Bip, and protein disulfide isomerase antibodies. Immunoblots were developed with the Amersham Corp. ECL system.

**Isolation of Microsomes**—RER microsomes containing reduced \(^{35}\)S-labeled HA were isolated from the rabbit reticulocyte lysate translation system by the centrifugation procedure described above. The pelleted microsomes were resuspended in a reticulocyte lysate mixture (52% rabbit reticulocyte lysate, 3.2 mM DTT, 0.02 MTris, pH 7.5) which was untreated or boiled at 100 °C for 10 min then centrifuged at 13,000 \(\times\) g for 10 min to remove precipitated macromolecules.

**RESULTS**

**The Lumenal Proteins of the Microsomes**—To translate and translocate the influenza HA in vitro, we used a standard reticulocyte lysate system in the presence of isolated dog pancreas microsomes. The mRNA of HA was obtained by in vitro transcription. Since our goal was to analyze the folding of translocated HA molecules in a system which would mirror the intact ER, it was important to first determine whether the chaperones and folding factors known to be resident in the lumen of the ER were present in our microsomal vesicles.

Microsomes stored in liquid nitrogen were thawed at 27 °C through a sucrose cushion. SDS-PAGE and Coomassie staining revealed that the majority of proteins were pelletable (Fig. 1A, second lane) with only a minor fraction of soluble, untrapped proteins remaining on top of the cushion (first lane). Next, lumenal proteins trapped inside the vesicles were released using a low concentration of nonionic detergent, Nikkol. While causing efficient lysis and release of internal proteins, the amount of detergent used was not sufficient to solubilize the membranes (Connolly et al., 1988; Kelleher et al., 1992). The third lane shows the released proteins and the fourth lane the pelletable membrane-associated proteins.
It was clear from the gel patterns that the membrane-associated proteins and the soluble lumenal proteins were almost quantitatively separated from each other after the mild detergent treatment. While the protein composition of the luminal proteins was relatively simple, the protein composition of the membrane fraction was exceedingly complex. The most abundant proteins in the microsome preparation, corresponded to four major luminal proteins. They were identified as GRP 94 (Mazzarella et al., 1987), BiP (Mazzarella et al., 1990), BiP/GRP78 (Haas et al., 1983; Pouyssegur et al., 1977), and protein disulfide isomerase (Freedman, 1984) by immunoblotting as indicated in the figure. These proteins are all ER chaperones and folding enzymes.

We concluded that the major ER folding factors were not only present in our microsome preparations at high concentrations, but also trapped inside the vesicles. The luminal contents was, moreover, dominated by a small group of folding factors present at high concentrations. All other luminal proteins including the endogenous substrate proteins (the pancreatic zymogens) were minor components of the ER. The protein composition suggested that the main function of the ER lumen in the pancreatic acinar cell is to promote efficient folding of disulfide-dependent proteins because both of these reducing agents are membrane permeable and cause reduction of the ER lumen. The problem can, however, be overcome by adding GSSG to the translation mixture. Using pancreatic zymogens and prolactin as model proteins, Scheele and Jacoby (1982) and Kaderbhai and Austen (1985) have shown that 1-3 mM GSSG in the translation mixture allows the oxidation of newly synthesized proteins inside the microsomes without severe inhibition of translation.

When we added 2 mM GSSG in the translation mixture, we too observed mobility changes in HA on non-reduced gels indicating disulfide oxidation (Fig. 2). After the 1-h translation period, the principal HA bands on non-reducing gels were UT and a set of three bands with mobilities faster than R. They were identical to two partially oxidized folding intermediates (IT1 and IT2) and fully oxidized HA (called NT for native) observed in live cells (Braakman et al., 1991). After reduction these three bands coalesced into a single band with a mobility equal to that of fully reduced R (Fig. 2, lower panel). As in live cells, a shift from IT1 to IT2 and further to NT occurred during a chase period in the presence of GSSG suggesting continued folding (30 and 60 min chase, Fig. 2, upper panel).

The smeared appearance of the UT band in the presence of GSSG suggested that it, too, underwent oxidation but in a heterogeneous manner. Correct folding of UT was not expected since glycosylation is needed for correct folding of HA in live cells (Gallagher et al., 1992; Hurtley et al., 1989). UT is presumably neither glycosylated nor processed by signal peptidease.

It is noteworthy that very few background bands were seen in these gels (Figs. 1 and 2). However, depending on the lysate and microsome preparation used, interfering background proteins could sometimes be observed. In such cases, immunoprecipitation was used to isolate the HA prior to SDS-PAGE.

**Translation and Translocation of HA—**HA mRNA was translated in the presence of these microsomes. After a 1-h translation period at 27 °C in the presence of Trans 35S label, 20 mM NEM was added to alkylate any free sulfhydryls, thus preventing further oxidation of newly synthesized HA. This alkylation procedure to quench the folding process (Braakman et al., 1991; Creighton, 1978) was used routinely for all samples in this study.

Two major labeled products were detected after SDS-PAGE: R and UT (Fig. 1B, first lane). Proteinase K digestion resulted in the degradation of UT, but R remained protected (Fig. 1B, second lane). R could be digested, however, if detergent was added (not shown). Since R had the same electrophoretic gel mobility as newly synthesized reduced HA in live cells, and since it immunoprecipitated with a variety of anti-HA antibodies, we concluded that it represented the translocated and core-glycosylated form of HA. The lack of R digestion using proteinase K suggested that R was translocated and that the microsomes were sealed.

UT migrated on the gel with an electrophoretic gel mobility equivalent to deglycosylated HA. Since it was also precipitable with anti-HA antibodies, it corresponded to an untranslocated and unglycosylated form of HA. The amount of labeled UT was variable between experiments.

**Cotranslational Folding of HA—**The redox conditions in a standard reticulocyte lysate translation protocol are designed to mimic the reducing environment in the cytosol and to optimize translation efficiency. DTT is typically present both in the lysate and in the microsomal preparation. In our mixture the DTT concentration was 2.8 mM. In addition, 2-mercaptoethanol at a final concentration of approximately 0.9 mM was present as a component of the Trans 35S label. While optimal for translation, such conditions do not allow the folding of disulfide-dependent proteins because both of these reducing agents are membrane permeable and cause reduction of the ER lumen. The problem can, however, be overcome by adding GSSG to the translation mixture. Using pancreatic zymogens and prolactin as model proteins, Scheele and Jacoby (1982) and Kaderbhai and Austen (1985) have shown that 1-3 mM GSSG in the translation mixture allows the oxidation of newly synthesized proteins inside the microsomes without severe inhibition of translation.

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It is noteworthy that very few background bands were seen in these gels (Figs. 1 and 2). However, depending on the lysate and microsome preparation used, interfering background proteins could sometimes be observed. In such cases, immunoprecipitation was used to isolate the HA prior to SDS-PAGE.

**Post-translational Folding of HA—**Given the relatively slow rate by which translation occurs in vitro, and the slow accumulation of labeled protein inside microsomes (Rothman and Lodish, 1977), the system described above was not ideal for analyzing the folding process. We needed a way to initiate the folding process more synchronously. For this, we copied a strategy previously developed for post-translational folding in intact cells. When synthesized in the presence of DTT, we have previously shown that HA remains trapped in the ER in an unfolded, reduced state (Braakman et al., 1992a, 1992b). When DTT is subsequently removed, it undergoes normal, synchronous folding post-translationally.

The corresponding experiment in microsomes involved the
vesicles (R) was first rapidly converted to IT1, then to IT2 translated in the presence of dog pancreas microsomes and 2 mM was used at a final bulk concentration of 3 mM, we found, as and after about 10 min, to be folded.

To determine the relative amount of IT1 and IT2 present, we took advantage of a series of antibodies (HC3, HC19, and HC100) which recognize the conformational epitopes A, B, and C, respectively, in the top domain of HA (Daniels et al., 1985, 1987; Hurtley et al., 1989). As previously shown for HA in live cells (Braakman et al., 1992a, 1992b), they all reacted with in vitro translated IT1, IT2, and NT.

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Translation of HA under the standard reducing conditions. The oxidizing agents were then added to the in vitro translation mixture, and the process of folding, thus synchronously initiated, could be followed during the chase. When GSSG was used at a final bulk concentration of 3 mM, we found, as shown in Fig. 3 (panel αHA), that the reduced HA in the vesicles (R) was first rapidly converted to IT1, then to IT2 and after about 10 min, to NT. Some aggregates containing HA were also seen at the top of the gel beginning at 10 min of chase.

Quantitative analysis of the various bands by densitometry, averaging three independent experiments (Fig. 4), showed that IT1 peaked at about 2 min after GSSG addition, and IT2 at about 5 min. The t1/2 of NT formation was approximately 7 min. The folding rate was thus approximately similar to that observed in live cells at 27 °C (Braakman et al., 1991). The pathway of post-translational folding was clearly R → IT1 → IT2 → NT. Although we have shown that IT1 and IT2 are precursors of NT during folding in living cells (Braakman et al., 1991), we have not previously been able to establish that IT1 is a precursor to IT2.

From the difference in radioactivity in HA bands before and after reduction prior to gel electrophoresis, we could estimate that the efficiency of folding was about 40%. The HA that was not correctly folded was present as disulfide-linked aggregates that did not efficiently enter the stacking gel (see Fig. 3). When the SDS-treated samples were reduced, the aggregated HA was monomerized and migrated with mobility equivalent to R (Fig. 3, lower panels). In live cells, we usually observe less than 10% of such misfolded, cross-linked aggregates (Hurtley et al., 1989), but in some cell types their amount can be as high as 50%. The efficiency of folding observed here is therefore lower than in most live cells but not dramatically different.

Folding Characterized by Conformation-specific Antibody Binding—To further analyze the post-translational folding process in microsomes, we took advantage of a series of conformation-specific antibodies against HA. F1 and F2 are monoclonals that recognize early and late folding intermediates of HA in lysates from live cells (Braakman et al., 1992a, 1992b). These epitopes are located in the stem domain. When used to precipitate the HA from alkylated and detergent-solubilized in vitro lysisates, F1 precipitated the aggregates, the reduced form (R), IT1, IT2, and the untranslocated form (UT), but not NT. In contrast, F2 antibodies precipitated only IT2 and NT. The progressive changes in reactivity observed for the chased samples with chase time mirrored those previously seen in cells (Braakman et al., 1992a). IT1, IT2, and NT recovered from microsomes were thus antigenically and electrophoretically equivalent to cellular folding intermediates.

We also used a series of antibodies (HC3, HC19, and HC100) which recognize the conformational epitopes A, B, and C, respectively, in the top domain of HA (Daniels et al., 1985, 1987; Hurtley et al., 1989). As previously shown for HA in live cells (Braakman et al., 1992a, 1992b), they all reacted with in vitro translated IT1, IT2, and NT.

Taking these results together, we concluded that the folding process in microsomes is similar in rate and overall pathway to that observed for HA in live cells. The efficiency was somewhat lower than in most cells but within an acceptable range.

Redox Effects on HA Folding—We found that GSSG supported proper post-translational folding over a wide concentration range (2–20 mM) with essentially constant kinetics and efficiency. A recent report has indicated that GSSG and GSH constitute the principal redox buffer inside the ER and that a carrier for the uptake of GSSG may exist in the ER membrane (Hwang et al., 1992).

To test whether folding could be induced by an artificial oxidizing agent, we tested diamide, a powerful, membrane-permeable sulphydryl oxidizing agent (Kosower and Kosower, 1987). We observed that it too promoted normal folding of HA (Fig. 5). However, unlike the natural oxidant, GSSG, diamide worked only within the narrow concentration range of 1.75–2.75 mM. At lower concentrations no folding could be observed, while higher concentrations induced misfolding and aggregation. Thus, unlike GSSG, high concentrations of diamide interfered with the fidelity of the folding process.

We measured the actual changes in redox potential in the reaction mixture upon addition of different GSSG and diamide concentrations, we used a reduction-oxidation microelectrode. As shown in Fig. 5, the starting redox potential in the in vitro translation mixture was −390 mV. When increasing concentrations of GSSG and diamide were added, the redox potential increased. For HA oxidation to start inside the microsomes, it had to rise above −310 mV. Proper folding of HA took place within the −310 to −210 mV potential range. While “over-oxidation” could not be induced with GSSG, it was easily achieved with diamide. It occurred when the redox potential exceeded −170 mV with 3.0 mM or more of diamide. Oxidized DTT, which is a weak oxidant, did not induce disulfide bond formation in this system even at concentrations up to 33 mM (−384 mV).

\(^2\)I. Braakman and A. Helenius, unpublished results.
FIG. 3. Post-translational folding of HA. 35S-Labeled HA was translated for 1 h at 27°C in the presence of 2.8 mM DTT and 0.9 mM 2-mercaptoethanol. At time 0, 3 mM GSSG was added to the lysate mixture. Samples were stopped at the above indicated times with 20 mM NEM and immunoprecipitated with αHA (polyclonal that recognizes all forms of HA), F1, and F2 (conformation-specific monoclonal) antibodies. Samples were analyzed by non-reducing (NR) and reducing (R) SDS-PAGE. Fluorographs of both resolving and stacking gels are shown.

Although useful for monitoring the optimal redox potential range in experiments employing GSSG and diamide, it was clear that the bulk redox potential was not the only factor that determined whether HA was oxidized or not. For example, when NAD and NADP were added as oxidants at concentrations as high as 20 and 30 mM, respectively, no HA folding could be observed although the recorded bulk redox potential should have been sufficient to initiate oxidation. It reached values of −297 and −305 mV, respectively. The reason for the lack of folding could have been that these oxidants may have been unable to penetrate the ER membrane efficiently. Since UT was also not oxidized in these samples, it was, however, more likely that these oxidants simply do not support efficient sulfhydryl oxidation under redox conditions comparable to GSSG and diamide.

Folding in Isolated Microsomes—All the experiments described above were performed in unfractionated in vitro translation mixtures. To separate the microsomes from the reticulocyte lysate after translation prior to oxidation, we subjected the mixture to centrifugation through a sucrose cushion in the presence of 1 mM DTT. The pelleted microsomes were then resuspended into fresh reticulocyte lysate containing 1–
Hemagglutinin Folding in Microsomes

5 mM GSSG or into boiled lysates that had been centrifuged to remove precipitates.

After resuspension into fresh reticulocyte lysate, HA folding was normal (Fig. 6, control). If, however, the microsomes were resuspended into a buffer containing different concentrations of GSSG, folding did not occur. Likewise, no folding occurred if GSSG was added to reticulocyte lysates that had been boiled to inactivate macromolecules (Fig. 6, boiled). Measurements of redox potentials for these boiled lysates showed slightly lower redox potentials at any given concentration of GSSG than observed in the untreated lysate. Still, the redox potentials at 3–5 mM GSSG were −302 and −289 mV and should therefore have been within the range needed for folding (Fig. 5). We concluded that some heat-sensitive component of the reticulocyte lysate was required to allow GSSG to oxidize microsomal HA.

Interestingly, normal folding occurred in the presence of boiled reticulocyte lysate when 1 mM diamide instead of GSSG was used (Fig. 6). This diamide concentration was below the range of diamide concentration found to allow oxidation in the reticulocyte lysate non-centrifuged experiments discussed above. Since diamide, unlike GSSG, is membrane permeable and does not require a carrier protein, the result suggested that heat-sensitive component(s) in the lysate were needed for GSSG transport into the microsomes. We fractionated the lysate by passing it through a filter with 10,000 Da cut-off, and found all the activity in the large molecular mass fraction, and none in the flow-through. This suggested that the activity in question was associated with macromolecules.

**Folding in Nikkol**—As already shown in Fig. 1, the nonionic detergent Nikkol can be used to release the soluble luminal proteins without solubilizing the microsomal membranes. After releasing the luminal proteins in this way, we suspended the membranes (which contained all the labeled HA) in fresh lysate and added GSSG. We found that GSSG no longer initiated proper folding of HA (Fig. 7). While oxidation did occur, it resulted in slow disulfide-linked HA aggregation. Some gradual degradation of the HA also took place. From this we concluded that the folding of HA in microsomes depends not only on a mildly oxidizing milieu but might also depend on the presence of luminal folding factors.

**DISCUSSION**

Among protein folding compartments in the eukaryotic cell, the ER is unique. It is topologically equivalent to the extracellular space, it is oxidizing, it has a massive capacity for protein maturation, it contains a high calcium ion concentration, and the products are primarily targeted for export and not for residence in the ER itself. Looking at the composition of the luminal proteins, the preponderance of folding factors is striking. At least in a secretory cell type such as the pancreatic acinar cell from which our microsomes were derived, the ER lumen seems to be almost exclusively devoted to a single function which is to assist the conformational maturation of newly synthesized proteins. The major folding factors, protein disulfide isomerase, BiP/GRP78, GRP94, and ERP72 are known to be present in almost millimolar concentrations in the ER (Hillson et al., 1984; Lyles et al., 1992a). They clearly out-number the substrate proteins present in this compartment.

For HA, the over-all efficiency of post-translational folding

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<tr>
<th>GSSG (mM)</th>
<th>diamide (mM)</th>
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![Fig. 6. Post-translational folding in isolated microsomes.](image)

Microsomes containing reduced 35S-labeled HA were isolated from the lysate mixture by ultracentrifugation through a sucrose cushion in the presence of 1 mM DTT. The pelleted microsomes were suspended in fresh or boiled lysate. Folding was initiated by the addition of 1–5 mM GSSG or 1 mM diamide at 27 °C. After 30 min, samples were alkylated with NEM and immunoprecipitated with aHA. Fluorographs of non-reducing (NR) and reducing (R) resolving and stacking SDS-PAGE gels are shown.

![Fig. 7. Post-translation folding in the absence of luminal proteins.](image)

35S-Labeled HA was translocated into dog pancreas microsomes under reducing conditions for 1 h at 27 °C. The 35S-labeled HA lysate mixture was treated with (+) and without (−) 0.05% Nikkol at 4 °C for 10 min. Post-translational folding of HA was initiated at time 0 by the addition of 3 mM GSSG at 27 °C. The time course of folding was studied as previously described in Fig. 3.
in microsomes was about 40%. As in live cells, the misfolded fraction was recovered in disulfide cross-linked aggregates which did not enter the SDS-PAGE resolving gel unless reduced (Hurtley et al., 1989; Marquardt et al., 1992). The efficiency was thus somewhat lower than normally observed in influenza infected cells, but similar to that of post-translational folding after prolonged DTT treatment (Braakman et al., 1992a). That post-translational folding becomes less efficient after extended DTT treatment may have to do with a progressive aggregation of reduced HA molecules in the ER as shown by recent experiments in live cells.

The post-translational folding of HA observed in microsomes was very similar to its folding in intact cells in terms of kinetics, the appearance of incompletely oxidized intermediates, and the progressive changes in conformational epitopes (Braakman et al., 1991, 1992a, 1992b). Folding began with rapid oxidation of HA’s top domain resulting in the transition from R to IT1 and the appearance of the “Top” domain epitopes A, B, and E. The formation of IT2 and NT (the fully oxidized HA) occurred more slowly. The transitions from IT1 to IT2 and IT2 to NT correspond, according to our unpublished results

\[ \text{formation of disulfides } \text{S-S in the hinge and } 14-466 \text{ in the stem region of the protein, respectively.} \]

Within the time span of a typical experiment, we did not observe any appreciable degradation of HA whether present as folding intermediates, oxidized products or misfolded aggregates. Such a slow degradation rate was consistent with our previous observations in live cells, where misfolded HA has a half-life of about 6 h (Hurtley et al., 1989).

As oxidants, we used several different agents of which GSSG and diamide were the only ones that supported proper folding of HA. GSSG is thought to be the main physiological redox buffering substance in the ER, and probably transported from the cytosol into the lumen through a membrane carrier (Hwang et al., 1992). Diamide is a membrane-permeable, synthetic, non-disulfide oxidant effective in oxidizing disulfides (Kosower and Kosower, 1987). Among the unsuccessful agents was oxidized DTT which was too weak to raise the redox potential in the lysate to a level that should have been high enough, but no HA oxidation was observed. This indicated, as already discussed by Wettlauer and co-workers (Wettlauer et al., 1987), that the redox potential does not necessarily parallel the efficiency by which oxidants support correct protein folding.

Presumably, the effectiveness of an oxidizing agent in the microsomal system depends both on its ability to penetrate into the lumen of the ER and its capacity to provide oxidizing equivalents to cysteine thiols. Once inside the lumen, the agent may directly oxidize the folding polypeptide chains or indirectly via redox enzymes present in the ER. While both diamide and GSSG are capable of oxidizing proteins directly (Kosower and Kosower, 1987; Saxena and Wettlauer, 1970), their primary effect in microsomes may be to oxidize protein disulfide isomerase or other redox enzymes, which in turn may catalyze the oxidation of the folding HA. Such an indirect mechanism is supported by the data of Bulleid and Freedman (1988), who were able to restore the folding of gamma-glutamyl protein disulfide isomerase-depleted microsomes by adding back oxidized protein disulfide isomerase without any redox buffer, and by our observation that GSSG was ineffective when added to opened microsomes devoid of luminal folding enzymes.

Although our redox potential measurements served as a useful operational reference in our studies, we do not think that they gave meaningful information as to the absolute redox potential inside the ER vesicles. Protein disulfide isomerase, ERp72, and ERp61, all of which have thioredoxin-like active sites, are present in near millimolar concentrations, providing substantial redox buffering capacity (Hillson et al., 1984; Lyles and Gilbert, 1992b). In addition, the likely presence of trapped glutathione, DTT, and 2-mercaptoethanol, makes it difficult to estimate what the internal redox potential might actually be.

In conclusion, we find that isolated microsomes provide a powerful system for studying protein folding. They contain a high concentration of luminal folding proteins, and they are active in translocation, co- and post-translational modification and in protein folding. So far, their capacity to fold proteins has only been exploited in a small number of studies (Bulleid et al., 1988; Kaderbhai et al., 1988; Lévy et al., 1991; Ribaudo and Margulies, 1992; Scheele et al., 1982). The combination of in vitro translation of recombinant mRNA and post-translational protein folding as developed for HA in this study provides an improved system for analyzing the role of folding factors and the folding milieu inside the ER. Since most secretory proteins and membrane glycoproteins contain critical disulfide bonds, it is probably applicable to a wide variety of proteins. One of the main advantages is that the folding process can be synchronously initiated by the addition of oxidizing agent. This makes the stepwise folding pathway taken by proteins under physiological or near-physiological conditions available for detailed analysis. Proteins which are normally synthesized in minute amounts can, moreover, be analyzed with adequate signal.

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