

Manipulating disulfide bond formation and protein folding in the endoplasmic reticulum

Ineke Braakman, Jonne Helenius and Ari Helenius

Department of Cell Biology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510-8002, USA

Communicated by J.Tooze

Addition of the reducing agent dithiothreitol (DTT) to the medium of living cells prevented disulfide bond formation in newly synthesized influenza hemagglutinin (HA0) and induced the reduction of already oxidized HA0 inside the ER. The reduced HA0 did not trimerize or leave the ER. When DTT was washed out, HA0 was rapidly oxidized, correctly folded, trimerized and transported to the Golgi complex. We concluded that protein folding and the redox conditions in the ER can be readily manipulated by addition of DTT without affecting most other cellular functions, that the reduced influenza HA0 remains largely unfolded, and that folding events that normally take place on the nascent HA0 chains can be delayed and induced post-translationally without loss in efficiency.

Key words: disulfide bonds/DTT/ER/reduction

Introduction

Most secretory proteins and membrane glycoproteins contain disulfide bonds. These cross-links, which can form within or between polypeptides, are often crucial for the function and stability of the folded proteins as well as for their maturation and intracellular transport. The disulfides are generated through oxidation in the endoplasmic reticulum (ER), which is the compartment where most folding and oligomeric assembly events take place. The ER lumen is unique among folding compartments in the eukaryotic cell because it provides a sufficiently oxidizing environment for disulfide formation as well as an enzyme, protein disulfide isomerase, to promote the formation of disulfide bonds (see Freedman, 1989). Since the ER is not the terminal compartment for most of the proteins targeted to it, a quality control system is present that limits exit from the ER to the Golgi complex to those proteins that are fully folded and oligomerized (Hurtley and Helenius, 1989). From the Golgi complex they are transported to various destinations inside and outside the cell. Misfolded, unassembled and incompletely oligomerized proteins are generally retained in the ER and eventually degraded.

For some multidomain proteins like IgG (Bergman and Kuehl, 1979), serum albumin (Peters and Davidson, 1982) and influenza hemagglutinin (HA0) (Braakman *et al.*, 1991) it has been shown that folding and disulfide bond formation begin on the nascent chain. The co-translational folding of a typical multidomain glycopolypeptide thus proceeds vectorially from the N-terminus towards the C-terminus.

When synthesis is completed, many of the disulfide bonds are already in place. Folding and disulfide bond formation continue post-translationally, resulting (usually within a few minutes) in a folded protein with a completely oxidized lumenal domain. Disulfide pairing during the folding process is, at least in part, catalyzed by protein disulfide isomerase (Bulleid and Freedman, 1988; Freedman, 1989).

In this paper, we have analyzed the effects of a reducing agent, dithiothreitol (DTT) on the folding of the influenza hemagglutinin in the ER of infected CHO15B cells. HA0 is a well-characterized membrane glycoprotein (84 kDa; 549 amino acids) with a large N-terminal moiety which folds in the ER lumen. The crystal structure of the ectodomain of the mature HA0 homotrimer shows a distinct domain structure with independently folded top and stem domains (Wiley and Skehel, 1987; Wilson *et al.*, 1981). The subunits in the trimer are not covalently associated, but each HA0 monomer contains six intrachain disulfide bonds, which are conserved in all influenza strains. Previous studies have shown that the protein is synthesized and co-translationally translocated into the ER lumen in 2 min (Braakman *et al.*, 1991). It folds and acquires disulfide bonds co- and post-translationally. The folded, oxidized monomers appear within a few minutes after chain termination and proceed to form trimers which are selectively transported to the cell surface via the Golgi complex (Copeland *et al.*, 1986; Gething *et al.*, 1986; Braakman *et al.*, 1991).

We found that, when added to living cells infected with influenza virus, DTT prevented the formation of disulfide bonds on nascent and newly synthesized HA0, and induced reduction of already folded and oxidized monomers. In their reduced form, the HA0 molecules were partially folded or unfolded and incapable of trimerization and intracellular transport. When the reducing agent was washed out, the reduced HA0 monomers became correctly oxidized and folded, indicating that the ER was able to re-establish oxidizing conditions rapidly, and that co-translational folding and disulfide bond formation were not required during HA0 folding.

Results

HA0 folding in unperturbed cells

To analyze the folding of HA0, we used a previously developed pulse-chase assay based on electrophoretic mobility differences between fully and partially oxidized HA0 (Braakman *et al.*, 1991). Conformational comparisons were made by immunoprecipitation with a panel of conformation-specific monoclonal and polyclonal antibodies. Typically, influenza infected CHO15B cells were labeled with [³⁵S]methionine and [³⁵S]cysteine for 2 min and chased for periods up to 60 min. At the end of each chase time, the cells were treated with a membrane-permeant alkylating agent, *N*-ethyl maleimide (NEM) to block free sulfhydryl groups and trap folding intermediates (Creighton, 1978). The

cells were then lysed and immunoprecipitated either with a polyclonal anti-HA0 antiserum that recognizes all conformational forms of the protein, or with different conformation-specific antibodies.

After a 2 min pulse and a 2 min chase, three HA0 bands were seen in non-reduced gels (Figure 1, lane 1). They correspond to untrimmed, fully oxidized HA0 (called NT) and to two incompletely (or incorrectly) oxidized forms, IT1 and IT2 (Braakman *et al.*, 1991). We have previously shown that IT1 and IT2 are intermediates in the folding process. During the chase they are converted to NT, which then trimerizes and moves to the Golgi complex, where its N-linked carbohydrates are trimmed. This results in a shift in the electrophoretic mobility both on reducing and non-reducing gels [G (for Golgi) in Figure 2], which is diagnostic for ER to Golgi transport of HA0 (Kornfeld and Kornfeld, 1985; Balch *et al.*, 1986; Braakman *et al.*, 1991, 1992).

Immunoprecipitations with conformation-specific antibodies revealed that IT1, IT2, NT and G were antigenically distinct (Table I). The formation of disulfide bonds was thus accompanied by a change in conformation, showing that we were assaying folding and not post-translational oxidation in an already folded protein. The amount of HA0 precipitated by each antibody was compared with the amount precipitated by the polyclonal anti-HA0 antiserum that recognizes all conformations of HA0 (Copeland *et al.*, 1986; Hurtley *et al.*, 1989; Braakman *et al.*, 1991). This provided a semi-quantitative estimate of immunoprecipitation efficiency.

The antibodies employed fell into four categories: (i) Conformation-specific antibodies to intermediates in the maturation process. These antibodies were called F1, F2, F3 and F4 (Table I). They all showed clearly distinguishable differences in reactivity with IT1, IT2, NT and G (Table I). Since F1, F2 and F3 were made against the HA2 subunit of mature hemagglutinin, the corresponding epitopes were located in the stem domain of HA0. Thus, the stem underwent remodeling during the post-translational folding period. (ii) Monoclonals against conformational epitopes in the top domain of mature HA0 molecules (HC3, HC19 and HC100) (Daniels *et al.*, 1984, 1987). The epitopes—called A, B and E—belong to the most dominant and best characterized antibody binding sites on the HA molecule (Wiley and Skehel, 1987). In addition to being present on the fully oxidized forms of the molecule (NT and G), they were strongly expressed in both folding intermediates IT1 and IT2. This suggested that the top domain reached a conformation similar to the final one early in the folding process, perhaps already co-translationally. (iii) A stem domain monoclonal specific for misfolded and acid-treated forms of HA0 (A1) (Copeland *et al.*, 1986, 1988; Hurtley *et al.*, 1989). It did not react with any of the folding intermediates, nor with the mature HA0. Misfolded HA0, which is sometimes seen as a side product of HA0 synthesis (Copeland *et al.*, 1988; Hurtley *et al.*, 1989), was therefore not detectable in infected CHO15B cells. Although this antibody reacts with misfolded cellular forms of HA0, it is strictly conformation dependent; it does not, for instance, precipitate SDS-denatured reduced HA0. (iv) A monoclonal antibody to BiP (Bole *et al.*, 1986). This antibody has been shown to precipitate BiP itself as well as complexes between BiP and misfolded or incompletely folded proteins (Bole *et al.*, 1986; Gething *et al.*, 1986; Dorner *et al.*, 1987; Kassenbrock *et al.*, 1988;

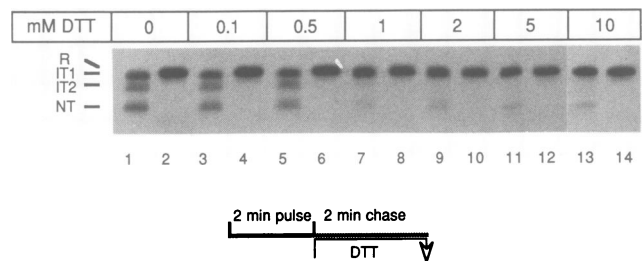


Fig. 1. Effect of different concentrations of DTT on HA0. CHO15B cells stably expressing HA0 were pulse-labeled for 2 min, followed by a chase of 2 min with or without different concentrations of DTT (0.1–10 mM). HA0 was immunoprecipitated from the lysates with polyclonal antiserum P, which recognizes all forms of the protein. Samples were analyzed by 7.5% SDS-PAGE under reducing (even lanes) and non-reducing (odd lanes) conditions. R is the completely reduced form of HA0.

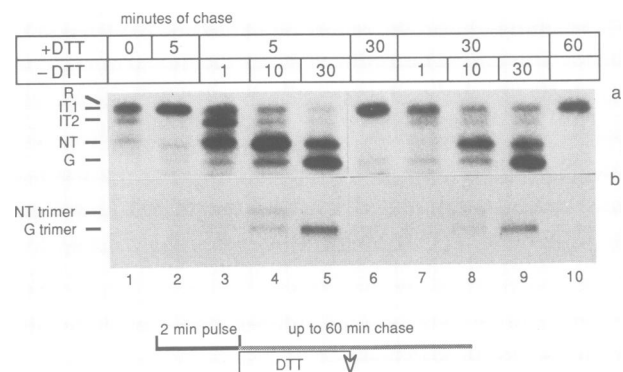


Fig. 2. Post-translational reduction and reoxidation of HA0. CHO15B cells were pulse-labeled for 2 min and chased for up to 60 min in the presence of 5 mM DTT (lanes 2, 6 and 10). The cells from lane 1 received one wash with DTT-containing medium. After 5 or 30 min incubation with DTT, the reducing agent was removed by two washes with normal chase medium followed by an incubation of up to 30 min without DTT (lanes 3–5 and 7–9). The detergent lysates were immunoprecipitated with the polyclonal antiserum P (A) and with the trimer-specific monoclonal antibody N2 (B), followed by 7.5% SDS-PAGE under non-reducing (A) or reducing (B) conditions. Long exposures were used for photographs. On shorter exposures, a precursor-product relationship between IT1, IT2 and NT has been established (Braakman *et al.*, 1991).

Kozutsumi *et al.*, 1988; Hurtley *et al.*, 1989). Misfolded HA0 is efficiently coprecipitated by this antibody (Hurtley *et al.*, 1989). Neither the folding intermediates nor NT or G, however, co-precipitated with anti-BiP (Table I) although care was taken to eliminate ATP from the lysates. This implied that BiP is not strongly associated with any of the normal maturation intermediates of the HA0 from the X31 strain of the influenza virus.

By monitoring the formation of disulfide bonds and conformation-dependent antigenic epitopes we could delineate some of the steps in the folding process. The same techniques provided us with the tools necessary to analyze the effects of agents such as DTT, which we expected to affect the folding process. Taken together, the results confirmed that, in unperturbed cells, the HA0 underwent a progressive co- and post-translational folding and oxidation process in the ER. Folding of the top domain occurred during translation while the stem domain acquired its folded form post-translationally. The disulfide bond formation

Table I. Conformation of different forms of HA0 as determined by immunoprecipitation

	F1 ^a	F2	F3	F4	HC3 (A)	HC19 (B)	HC100 (E)	A1	anti-BiP
HA0 intermediates in the unperturbed cell									
1 IT1	+++ ^b	-	+	+++	++	+++	++	-	-
2 IT2	-	++	++	++	++	+++	++	-	-
3 NT	-	++	++	±	+++	+++	+++	-	-
4 G	-	-	+	-	+++	+++	+++	-	-
HA0 post-translationally reduced									
5 R ^c (3 min chase + DTT)	++	-	++	+++	++	++	++	-	-
6 R (30 min chase + DTT)	+++	-	+++	+++	+	+	±	-	-
7 R (5 min chase + DTT - ATP)	+++	nd ^d	nd	nd	++	++	++	++	+
HA0 after translation in the presence of DTT									
8 R (no chase)	+++	-	+++	+++	+	+	+	-	-
9 R (30 min chase + DTT)	+++	-	+++	+++	+	+	±	-	-
10 R (5 min chase + DTT - ATP)	+++	nd	nd	nd	-	-	-	+++	+
In vitro reduced HA0									
11 R ^e	++	-	++	++	-	-	-	-	-

^aAntibodies are described in Materials and methods.

^b±, <10%; +, 10–40%; ++ 40–80%; +++, >80%; where the amount immunoprecipitated by the polyclonal antiserum P is 100%.

^cR: any reduced form of HA0.

^dnd: not determined.

^eThe *in vitro* reduced and denatured HA0 was prepared as described in Materials and methods.

started on the nascent chains, because no full-length HA0 comigrated with R, the completely reduced form of HA0 (Braakman *et al.*, 1991).

Reduction of HA0 in the ER

To test whether it was possible to reduce newly synthesized HA0 in the ER of living cells, we pulsed cells for 2 min and then incubated them with different concentrations of DTT (0.1–10 mM) for 2 min at 37°C. Analysis of non-reduced samples by SDS-PAGE showed that ≥ 1 mM DTT was needed to reduce HA0 in the ER (Figure 1, lane 7). At 1 mM DTT, NT and IT2 were reduced to forms that comigrated with IT1 and with R, the fully reduced form of HA0 which is obtained after SDS denaturation and DTT reduction at 95°C. R migrated slightly more slowly than IT1 on non-reduced gels (lanes 1 and 2). After longer incubation with 1 mM DTT, or after 2 min incubation with ≥ 5 mM DTT (lanes 11–14), HA0 was completely converted to a form comigrating with R. Thus, the addition of DTT resulted in efficient reduction of fully and partially oxidized HA0 molecules in the ER. Irrespective of the DTT concentration used, a small fraction of HA0 always resisted reduction. Preliminary results suggest that this fraction corresponds at least in part to trimeric HA0 (Utpal Tatu, I. Braakman and A. Helenius).

Post-translational reduction of the labeled HA0 was very fast: a short wash with 5 mM DTT was sufficient to reduce part of the protein (Figure 2, lane 1). As long as DTT was present, the HA0 remained reduced. Judging by the inability to react with the trimer-specific antibody N2 (Figure 2B, lanes 2, 6 and 10), it failed to trimerize and was neither transported to the Golgi complex (Figure 2A and B, lanes 2, 6 and 10) nor rapidly degraded. Similar reduction effects were observed with DTT concentrations as low as 1 mM, but as already mentioned, the initial reduction was somewhat slower. When the weaker reductor, 2-mercaptoethanol, was added at a concentration of 5 mM, only minor effects on the oxidation state of HA0 were observed.

Immunoprecipitation with our antibody panel showed that

most of the detectable conformational features of the folded HA0 were lost upon DTT addition. The conformation of the reduced HA0 approached that of the denatured, reduced HA0 produced *in vitro* (Table I, line 11). Like the denatured HA0, the *in vivo* reduced HA0 was not precipitated with A1, the antibody that recognizes HA0 in its previously characterized, cell-associated misfolded forms (Copeland *et al.*, 1988; Hurtley *et al.*, 1989). It was recognized by F1, F3 and F4, the earliest detectable antibody binding sites in the stem, but not by F2 which appears in IT2 and NT. However, unlike the *in vitro* denatured HA0, it was precipitated, albeit weakly, by antibodies to the epitopes in the top domain. Although these epitopes were gradually lost during incubation with the reducing agent, they remained partially recognized in the final reduced HA0 incubated for 30 min in DTT (Table I, line 6).

From this we concluded that both the stem and the top domains of HA0 unfolded extensively when the molecule was reduced in the ER. The loss of the A, B and E epitopes after DTT addition was probably caused by reduction of the two internal disulfide bonds 97–139 and 64–76 in the top domain of HA0. These disulfides are located close to epitopes A and E, respectively, and are likely to contribute to the conformation of the antigen binding sites, which constitute loops on the surface of the molecule. These epitopes were completely absent in denatured HA0 (Table I, line 11), but present in cell-associated, misfolded forms of HA0 (Hurtley *et al.*, 1989).

Although this conformational analysis leaves many questions unanswered, it suggests that the HA0 molecules in the ER do not adopt a stable conformation in the absence of disulfide bonds (Table I, lines 5 and 6). They may remain largely unfolded, poised in conformations intermediate between a fully denatured protein (Table I, line 11) and the first detectable full-length folding intermediate, IT1 (Table I, line 1).

Interestingly, when cells were treated with ATP-depleting metabolic inhibitors (sodium azide and deoxyglucose in glucose-free medium), the reduced HA0 molecules under-

went a conformational alteration. BiP-HA0 complexes could now be recovered by co-precipitation, and the HA0 was recognized by antibody A1 (Table I, line 7). This implied that the presence of ATP influenced the conformation adopted by HA0 molecules inside the ER; without ATP, they expressed properties similar to 'misfolded' HA0. ATP seemed to prevent the reduced proteins from getting trapped into a 'misfolded' conformation, a role that is consistent with our observations that, in the absence of DTT, HA0 folding in the ER as well as the maintenance of HA0's folded structure within that compartment, is dependent on metabolic energy (Braakman *et al.*, 1992).

Reoxidation of reduced HA0

When the DTT was washed away, rapid reoxidation of HA0 occurred. Within a minute, folding intermediates IT1 and IT2 reappeared, and NT started to be formed. The folded HA0 soon trimerized, and was transported to the Golgi complex, where its N-linked carbohydrates were trimmed, resulting in the faster moving band labeled G (Figure 2A and B, lanes 3–5 and 7–9). This implied that oxidizing conditions were rapidly re-established in the ER lumen, allowing normal folding and maturation. As far as the formation of disulfide bonds was concerned, the folding process was now entirely post-translational. Evidently, the outcome of the folding process did not depend on disulfides being formed co-translationally although disulfides normally do appear already on the nascent chains (Braakman *et al.*, 1991).

The rate of maturation after DTT wash-out was similar to that of HA0 in unperturbed control cells (Braakman *et al.*, 1991, 1992). We estimated that the time needed for half of the labeled HA0 to reach the fully oxidized form (NT) was ~3 min, and that for half of the protein to reach the medial Golgi 15–20 min. The efficiency of folding was >95% (Figure 3, lane 2). However, if the DTT incubation was prolonged to ≥30 min, a fraction of HA0 failed to mature and the folding rate gradually decreased. Fewer oxidized HA0 monomers appeared and disulfide-cross-linked complexes were formed (Figure 3, lanes 4 and 6). These ran as aggregates in non-reduced gels and as monomers after SDS denaturation and reduction (not shown). The aggregated HA0 persisted over several hours, indicating that it represented an irreversible diversion from the correct folding pathway. The increase in misfolding after prolonged DTT treatment suggested that slow changes in the reduced proteins may take place, rendering some of the HA0 molecules less capable of correct post-translational folding. Alternatively, some of the reduced proteins may have been slowly separated from the folding machinery within the ER, or the folding machinery itself may have been slowly inactivated by DTT. The possibility that the re-establishment of oxidative conditions in the ER lumen was affected by prolonged DTT treatment was unlikely because disulfide bonds appeared equally quickly after long and short reduction periods.

Synthesis of HA0 in the presence of DTT

Since the refolding and maturation of HA0 after DTT removal could have been facilitated by partial retention of secondary and tertiary structure in the artificially reduced HA0, we determined what would happen if disulfides were not allowed to be formed during translation. For this

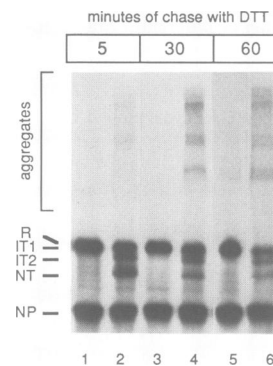


Fig. 3. Reoxidation after longer DTT treatment increases the misfolded HA0 fraction. The experimental protocol was as described in Figure 2. After a 2 min pulse, the cells were chased for 5, 30 or 60 min with 5 mM DTT (lanes 1, 3 and 5, respectively). This was followed by an incubation of 5 min without DTT (lanes 2, 4 and 6, respectively). The detergent lysates were immunoprecipitated with the polyclonal antiserum P and analyzed by non-reducing 7.5% SDS-PAGE. NP is the nucleoprotein of influenza, for reference of the amount of sample in each lane.

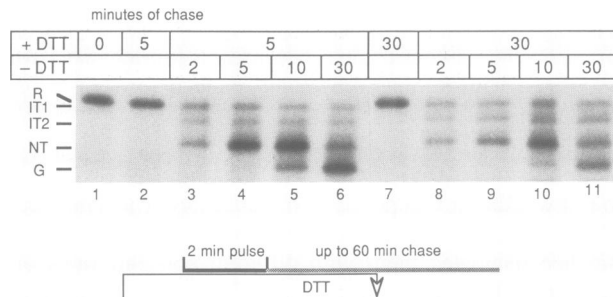


Fig. 4. Post-translational folding of HA0 translated in the presence of 5 mM DTT. The experimental protocol was as in Figure 2A, except that 5 mM DTT was also present during and 5 min before the pulse. Long exposures were used for photographs. On shorter exposures, a precursor-product relationship between IT1, IT2 and NT has been established before (Braakman *et al.*, 1991).

purpose, DTT was added 5 min before the radioactive pulse, and kept present throughout the pulse and the chase.

The amounts of radioactive methionine and cysteine incorporated into HA0 after 5 min incubation with 5 mM DTT were found to be only slightly (<5%) lower than in the control cells, whereas HA0 translation, translocation and covalent processing were unaffected. Only after longer incubation with 5 mM DTT did we see a significant inhibition of translation (not shown), while 5 min with 50 mM DTT completely abolished incorporation of radioactive label into the protein. We found that the HA0 molecules synthesized in the presence of DTT comigrated with fully reduced HA0 (R) (Figure 4, lane 1). Antigenically, they were identical to the post-translationally reduced HA0 after 30 min reduction (Table I, lines 6, 8 and 9). The expression of epitopes A, B and E in the top domain, for example, was again detectable but marginal. Thus, synthesis of HA0 in the presence of DTT resulted in a protein similar or identical to the HA0 obtained after extensive post-translational reduction within the living cell. We concluded from this that, in the absence of any disulfide bonds, newly synthesized HA0 was still capable of obtaining some conformational features that are present in the native protein.

When the DTT was washed out, the HA0 oxidized to IT1, IT2 and NT, and proceeded to fold, trimerize and exit from the ER normally (Figure 4, lanes 3–6 and 8–11). The rate was somewhat lower than for post-translationally reduced HA0 but the efficiency was equally high. This result demonstrated that a polypeptide synthesized without co-translational disulfide bonds acquired a partially folded conformation and that it could complete its folding process post-translationally when oxidizing conditions were restored.

Discussion

The addition of a strong reducing agent, DTT, was found to have a profound effect on the folding of HA0 in the ER. While the protein was translated, translocated and processed normally, intra-molecular disulfide bonds did not form. Not only did the HA0 synthesized in the presence of DTT remain reduced, but previously synthesized monomers which had already acquired a partial or a full complement of disulfides were also reduced.

The reduced HA0 in the ER represented a form not normally found as a precursor during HA0 biogenesis (Braakman *et al.*, 1991). Conformationally and functionally, it displayed characteristics most similar to completely denatured and reduced HA0, except for the partial expression of the prominent top domain epitopes A, B and E. This form of the protein might be similar in its overall properties to the 'molten globule' state observed during *in vitro* refolding studies (Ptitsyn, 1987; Kuwajima, 1989; Martin *et al.*, 1991). It did not trimerize and was not transported from the ER to the Golgi complex, consistent with previous results indicating that only trimers are transport-competent (Copeland *et al.*, 1986, 1988; Gething *et al.*, 1986). In contrast to our DTT effects, Alberini *et al.* (1990) found that 5 mM 2-mercaptoethanol allowed secretion of otherwise retained IgM assembly intermediates. This milder reducing agent may only have reduced the exposed tail-cysteine responsible for retention, but not the internal disulfides of the protein. In our hands, it had only a minor effect on HA0 in the ER.

When the DTT was washed out, the disulfides in HA0 were rapidly and correctly established. The protein acquired the correct epitopes and trimerized. While completely reduced HA0 is not normally an intermediate inside the cell (Braakman *et al.*, 1991), both the protein and the folding machinery had no problems in reproducing the correct disulfide pairing and the correct folding process post-translationally from fully reduced non-physiological starting material. Evidently, folding and disulfide pairing of HA0 did not need to be vectorial to be accurate. This implies that kinetic influences do not play a decisive role in the folding process (see Jaenicke, 1991). Such influences could arise from the vectorial nature of protein biosynthesis—since a polypeptide chain is translated from N- to C-terminus—, from discontinuities in the translation rate, from temporary associations with molecules in the translocation apparatus or with enzymes that catalyze co-translational modifications such as glycosylation and signal sequence removal. The folding of HA0 is therefore more likely to be determined by thermodynamic constraints (see Jaenicke, 1991). Showing a lack of difference between HA0 folding occurring co- or post-translationally, our results provide justification for the use of *in vitro* refolding experiments as a model for

intracellular folding events. While making use of denatured full-length proteins as a starting material, *in vitro* refolding experiments have provided most of our present insights into the biochemistry and biophysics of protein folding. The refolding process in this approach is obviously by definition post-translational.

So far, we have analyzed the effects of DTT on three other proteins and have not seen any instance where a protein could not be reduced and reoxidized in the ER. These proteins were the vesicular stomatitis virus G protein (seven disulfide bonds) and glycoproteins E1 and p62 of Semliki forest virus with 16 and 18 cysteines in the ectodomain each (I. Braakman, Aravinda De Silva, Thorsten Marquardt and A. Helenius, unpublished experiments). They all could be synthesized without disulfide bonds and displayed normal oxidation and folding after DTT wash-out. In the case of Semliki forest virus glycoproteins, the folding efficiency was only partial, resembling that observed for HA0 after long incubations with DTT.

The role of disulfide bonds during refolding of proteins *in vitro* or folding *in vivo* has been extensively analyzed and reviewed (Creighton, 1986, 1988; Jaenicke, 1987, 1991). Their importance in the folding process varies (Pace *et al.*, 1988; Taniyama *et al.*, 1990; Bardwell *et al.*, 1991; Doig and Williams, 1991). In many cases the disulfide bonds are necessary to stabilize the folded form of a protein without directly affecting the folding process (Long *et al.*, 1990). In other cases, key disulfide bonds play a direct role in folding (Creighton, 1986, 1988; Karnik *et al.*, 1988; Saganuma *et al.*, 1989; Ortega *et al.*, 1991). In the case of HA0 in the ER of living cells, some folding can apparently occur without disulfide bonds, but the protein cannot acquire a correctly folded top domain or a trimerization-competent monomeric structure.

Somewhat surprisingly, DTT did not seem to have serious adverse effects on translation, translocation, N-glycosylation or signal sequence removal. Preliminary results have shown that the secretory pathway and the Golgi complex continue to function in the presence of 5 mM DTT (Utpal Tatu, I. Braakman and A. Helenius, manuscript in preparation). The lack of general inhibitory effects suggested that disulfide bonded proteins are not critical in these processes, perhaps because most of them are cytosolic and therefore already adapted to more reducing conditions. Moreover, known resident luminal ER proteins contain fewer cysteines than secretory proteins, which may allow the former proteins to function in a compartment alongside with potent disulfide modifying activities.

That DTT can be used to delay the maturation process and to accumulate unfolded or partially folded molecules in the ER has proven useful for analyzing other aspects of folding. We have recently taken advantage of this to determine whether the folding process is energy dependent (Braakman *et al.*, 1992). Our results showed that when cells were depleted of ATP, the folding process was dramatically disturbed. HA0 was recovered in large disulfide cross-linked aggregates. Not only newly synthesized, unfolded proteins, but also already folded monomers which were misfolded upon energy depletion, got trapped into these aggregates.

The ER lumen differs from the cytoplasm in that it is more oxidizing. How these conditions are maintained is not clear, but oxidized glutathione from the cytosol may play a role (Scheele and Jacoby, 1982, 1983). Our results showed that

the redox conditions could be rapidly modified by externally added DTT, and that they were restored almost instantly when the DTT was removed. It will be of interest to analyze the mechanisms by which the oxidizing conditions in the ER are established and maintained.

Materials and methods

Cells and virus

We used a CHO cell line, developed by Dr D.Wiley, that expresses X31 HA0 constitutively or CHO15B cells 6 h after infection with X31 influenza virus (derived from A/Aichi/1968/H3N2). The CHO15B cells were used in most experiments because they are defective in *N*-acetylglucosaminyltransferase I (Gottlieb *et al.*, 1975; Balch *et al.*, 1986) and the trimmed Man₅GlcNAc₂Asn form of the N-linked carbohydrates cannot be processed further. This allows easy distinction between pre-Golgi (NT) and later (G) forms of HA0 by SDS-PAGE.

Folding of HA0

The folding of HA0 was followed as described before (Braakman *et al.*, 1991). Cells expressing HA0 were pulse-labeled for 2 min with 125 μ Ci/ml of [³⁵S]cysteine and Tran[³⁵S]label and chased with 5 mM methionine, 5 mM cysteine and 0.5 mM cycloheximide to block elongation of labeled nascent chains. Ice-cold PBS with 20 mM *N*-ethylmaleimide was added at the end of the chase to prevent further rearrangement of disulfide bonds. This concentration of *N*-ethylmaleimide was at least 10-fold the concentration needed to preserve IT1 and IT2 consistently. The cells were lysed in ice-cold 0.5% Triton X-100 in MNT (20 mM MES, 100 mM NaCl, 30 mM Tris-HCl, pH 7.5) containing 20 mM *N*-ethylmaleimide, 1 mM EDTA, 1 mM PMSF and 10 μ g/ml each of chymostatin, leupeptin, antipain and pepstatin. To follow the maturation of the disulfide bonds in HA0, the protein was immunoprecipitated from the lysates with the polyclonal rabbit antiserum (P) against X31 virus, which recognizes all forms of the hemagglutinin (Copeland *et al.*, 1986; Hurlley *et al.*, 1989; Braakman *et al.*, 1991). Fluorographs were then made from reducing and non-reducing SDS-PAGE (7.5%) in minigels.

To study the conformation of the different forms of HA0, a variety of antibodies was used to immunoprecipitate the protein from the lysates. The *in vitro* denatured and reduced HA0 (Table I, line 11) was prepared from lysates of cells pulse-labeled for 2 min and chased for 5 min without DTT. SDS and DTT were added to these lysates to final concentrations of 0.4% and to 20 mM respectively, and the reaction was then incubated for 5 min at 95°C. The sample was then cooled, quenched with 50 mM *N*-ethylmaleimide, diluted with Triton X-100 to a final concentration of 1.8% Triton X-100 and 0.06% SDS and used for immunoprecipitation.

The antibodies used were from various sources. F1 and F2 are mouse monoclonal IgGs made against non-denatured HA2 (Braakman *et al.*, 1991). F3 is a rabbit polyclonal antiserum made against non-denatured HA2, and F4 is a rabbit polyclonal made against SDS-denatured HA0, eluted out of a non-reducing SDS-polyacrylamide gel. Precipitations with F3 and F4 were carried out as done for the polyclonal rabbit antiserum P (Braakman *et al.*, 1991). The mouse monoclonal antibodies HC3, HC19 and HC100, mapped against epitopes A, B and E, respectively, all in the top domain of HA0, were described before (Daniels *et al.*, 1984, 1987; Wiley and Skehel, 1987; Hurlley *et al.*, 1989). A1 is a mouse monoclonal antibody that recognizes misfolded forms of HA0 (Copeland *et al.*, 1986; Hurlley *et al.*, 1989); it was made against acid-treated HA0. The mouse monoclonal antibody N2 is specific for HA0 trimers (Copeland *et al.*, 1986). The rat monoclonal antibody to BiP (Haas and Wabl, 1983) precipitates native BiP (Bole *et al.*, 1986) as well as complexes between BiP and misfolded HA0 (Bole *et al.*, 1986; Gething *et al.*, 1986; Dörner *et al.*, 1987; Kassenbrock *et al.*, 1988; Kozutsumi *et al.*, 1988; Hurlley *et al.*, 1989). In the immunoprecipitation protocol, care was taken to optimize coprecipitation of BiP-associated proteins (Hurlley *et al.*, 1989).

Reduction and reoxidation of HA0

For post-translational reduction, DTT was added to the chase medium in the indicated concentrations. For synthesis in the presence of DTT, the reducing agent was added 5 min before as well as during the pulse, and during the chase as indicated. Care was taken that all cells received the same concentration of DTT for the same time before the pulse started. For reoxidation, DTT was removed by two changes of chase medium without DTT followed by an incubation in regular chase medium as described above.

ATP depletion

Where indicated, the cells were depleted of ATP by incubation in glucose-free DMEM containing 20 mM 2-deoxy-D-glucose and 10 mM sodium azide (Braakman *et al.*, 1992).

Acknowledgements

We thank Drs J.Skehel, R.Webster and D.Bole for antibodies, and H.Tan for photography. We thank members of the Mellman-Helenius laboratory for advice and criticism. This work was supported by grants from the National Institutes of Health to Ari Helenius. Ineke Braakman was supported by a post-doctoral fellowship from the Human Frontier Science Program Organization.

References

- Alberini, C.M., Bet, P., Milstein, C. and Sitia, R. (1990) *Nature*, **347**, 485–487.
- Balch, W.E., Elliott, M.M. and Keller, D.S. (1986) *J. Cell Biol.*, **261**, 14681–14689.
- Bardwell, J.C.A., McGovern, K. and Beckwith, J. (1991) *Cell*, **67**, 581–589.
- Bergman, L.W. and Kuehl, W.M. (1979) *J. Biol. Chem.*, **254**, 8869–8876.
- Bole, D.G., Hendershot, L.M. and Kearney, J.F. (1986) *J. Cell Biol.*, **102**, 1558–1566.
- Braakman, I., Hoover-Litty, H., Wagner, K.R. and Helenius, A. (1991) *J. Cell Biol.*, **114**, 401–411.
- Braakman, I., Helenius, J. and Helenius, A. (1992) *Nature*, **357**, 260–262.
- Bulleid, N.J. and Freedman, R.B. (1988) *Nature*, **335**, 649–651.
- Copeland, C.S., Doms, R.W., Bolzau, E.M., Webster, R.G. and Helenius, A. (1986) *J. Cell Biol.*, **103**, 1179–1191.
- Copeland, C.S., Zimmer, K.-P., Wagner, K.R., Healey, G.A., Mellman, I. and Helenius, A. (1988) *Cell*, **53**, 197–209.
- Creighton, T.E. (1978) *Prog. Biophys. Mol. Biol.*, **33**, 231–297.
- Creighton, T.E. (1986) *Methods Enzymol.*, **131**, 83–106.
- Creighton, T.E. (1988) *BioEssays*, **8**, 57–62.
- Daniels, R.S., Douglas, A.R., Skehel, J.J., Wiley, D.C., Naeve, C.W., Webster, R.G., Rogers, G.N. and Paulson, J.C. (1984) *Virology*, **138**, 174–177.
- Daniels, R.S., Jeffries, S., Yates, P., Schild, G.C., Rogers, G.N., Paulson, J.C., Wharton, S.A., Douglas, A.R., Skehel, J.J. and Wiley, D.C. (1987) *EMBO J.*, **6**, 1459–1465.
- Doig, A.J. and Williams, D.H. (1991) *J. Mol. Biol.*, **217**, 389–398.
- Dörner, A.J., Bole, D.G. and Kaufman, R.J. (1987) *J. Cell Biol.*, **105**, 2665–2674.
- Freedman, R.B. (1989) *Cell*, **57**, 1069–1072.
- Gething, M.-J., McCammon, K. and Sambrook, J. (1986) *Cell*, **46**, 939–950.
- Gottlieb, C., Baenziger, J. and Kornfeld, S. (1975) *J. Biol. Chem.*, **250**, 3303–3309.
- Haas, I.G. and Wabl, M. (1983) *Nature*, **306**, 387–389.
- Hurlley, S.M. and Helenius, A. (1989) *Annu. Rev. Cell Biol.*, **5**, 277–307.
- Hurlley, S.M., Bole, D.G., Hoover-Litty, H., Helenius, A. and Copeland, C.S. (1989) *J. Cell Biol.*, **108**, 2117–2126.
- Jaenicke, R. (1987) *Prog. Biophys. Mol. Biol.*, **49**, 117–237.
- Jaenicke, R. (1991) *Biochemistry*, **30**, 3147–3161.
- Karnik, S.S., Sakmar, T.P., Chen, H.-B. and Khorana, H.G. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 8459–8463.
- Kassenbrock, C.K., Garcia, P.D., Walter, P. and Kelly, R.B. (1988) *Nature*, **333**, 90–93.
- Kornfeld, R. and Kornfeld, S. (1985) *Annu. Rev. Biochem.*, **54**, 631–664.
- Kozutsumi, Y., Segal, M., Normington, K., Gething, M.-J. and Sambrook, J. (1988) *Nature*, **332**, 462–464.
- Kuwajima, K. (1989) *Protein Struct. Funct. Genet.*, **6**, 87–103.
- Long, D., Cohen, G.H., Muggenridge, M.I. and Eisenberg, R.J. (1990) *J. Virol.*, **64**, 5542–5552.
- Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A.L. and Hartl, F.-U. (1991) *Nature*, **352**, 36–42.
- Ortega, S., Schaeffer, M.-T., Soderman, D., DiSalvo, J., Linemeyer, D.L., Gimenez-Gallego, G. and Thomas, K.A. (1991) *J. Biol. Chem.*, **266**, 5842–5846.
- Pace, C.N., Grimsley, G.R., Thomson, J.A. and Barnett, B.J. (1988) *J. Biol. Chem.*, **263**, 11820–11825.
- Peters, T., Jr and Davidson, L.K. (1982) *J. Biol. Chem.*, **257**, 8847–8853.
- Ptitsyn, O.B. (1987) *J. Protein Chem.*, **6**, 272–293.
- Scheele, G. and Jacoby, R. (1982) *J. Biol. Chem.*, **257**, 12277–12282.
- Scheele, G. and Jacoby, R. (1983) *J. Biol. Chem.*, **258**, 2005–2009.
- Suganuma, N., Matzuk, M.M. and Boime, I. (1989) *J. Biol. Chem.*, **264**, 19302–19307.
- Taniyama, Y., Yamamoto, Y., Kuroki, R. and Kikuchi, M. (1990) *J. Biol. Chem.*, **265**, 7570–7575.
- Wiley, D.C. and Skehel, J.J. (1987) *Annu. Rev. Biochem.*, **56**, 365–395.
- Wilson, I.A., Skehel, J.J. and Wiley, D.C. (1981) *Nature*, **289**, 366–373.

Received on December 5, 1991; revised on January 27, 1992