The Number and Location of Glycans on Influenza Hemagglutinin Determine Folding and Association with Calnexin and Calreticulin

Daniel N. Hebert, Jian-Xin Zhang, Wei Chen, Brigitte Foellmer, and Ari Helenius
Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06520-8002

Abstract. Calnexin and calreticulin are homologous molecular chaperones that promote proper folding, oligomeric assembly, and quality control of newly synthesized glycoproteins in the endoplasmic reticulum (ER). Both are lectins that bind to substrate glycoproteins that have monoglycosylated N-linked oligosaccharides. Their binding to newly translated influenza virus hemagglutinin (HA), and various mutants thereof, was analyzed in microsomes after in vitro translation and expression in live CHO cells. A large fraction of the HA molecules was found to occur in ternary HA–calnexin–calreticulin complexes. In contrast to calnexin, calreticulin was found to bind primarily to early folding intermediates. Analysis of HA mutants with different numbers and locations of N-linked glycans showed that although the two chaperones share the same carbohydrate specificity, they display distinct binding properties; calreticulin binding depends on the oligosaccharides in the more rapidly folding top/hinge domain of HA whereas calnexin is less discriminating. Calnexin’s binding was reduced if the HA was expressed as a soluble anchor-free protein rather than membrane bound. When the co- and posttranslational folding and trimerization of glycosylation mutants was analyzed, it was observed that removal of stem domain glycans caused accelerated folding whereas removal of the top domain glycans (especially the oligosaccharide attached to Asn81) inhibited folding. In summary, the data established that individual N-linked glycans in HA have distinct roles in calnexin/calreticulin binding and in co- and posttranslational folding.

The ER of most eukaryotic cells contains two homologous lectin-like chaperones called calnexin and calreticulin. Calnexin is a membrane protein and calreticulin a soluble luminal protein. They interact transiently with a variety of newly synthesized glycoproteins by attaching to partially trimmed N-linked oligosaccharide moieties carrying a single glucose residue in the α1–3 antenna (Ou et al., 1993; Hammond and Helenius, 1994; Hammond et al., 1994; Hebert et al., 1995; Peterson et al., 1995; Teichert and Salter, 1995; Ware et al., 1995; Spiro et al., 1996). The association of these chaperones with their substrate glycoproteins promotes correct folding and oligomeric assembly, prevents degradation, and supports quality control (Rajagopalan and Brenner, 1994; Hebert et al., 1996; Vassilakos et al., 1996).

When transferred to growing nascent chains, the N-linked core oligosaccharides carry three glucoses. ER glucosidases I and II rapidly remove two of them, thus generating the monoglycosylated forms (Glc1Man7,9NAcGlc2) that serve as ligands for calnexin and calreticulin binding. The remaining single glucose residue is subsequently removed by glucosidase II, resulting in the dissociation of the chaperone complex (Hebert et al., 1995, 1996; Rodan et al., 1996; Van Leeuwen and Kearse, 1996). The monoglycosylated form of the oligosaccharides is also generated in the ER by the action of UDP-glucose/glycoprotein glucosyltransferase. This luminal enzyme selectively reglucosylates glycoproteins that possess high mannose glycans only if the proteins are incompletely folded (Sousa et al., 1992; Trombetta and Parodi, 1992). Thus, by adding and removing glucoses, glucosidase II and the glucosyltransferase drive their substrates through a cycle of calnexin/calreticulin binding and release (Hammond and Helenius, 1993; Hebert et al., 1995; Van Leeuwen and Kearse, 1997). Glycoproteins stay in the cycle as long as they have a nonnative conformation, with the glucosyltransferase serving as a folding sensor (Suh et al., 1989; Hammond et al., 1994).

Although identical in their oligosaccharide specificity (Hammond et al., 1994; Peterson et al., 1995; Ware et al., 1995; Spiro et al., 1996), recent studies suggest that calnexin and calreticulin may differ in their substrate selection.
Vesicular stomatitis virus G protein binds to calnexin but not to calreticulin (Hammond and Helenius, 1994; Peterson et al., 1995). The bound proteins observed by coimmunoprecipitation from pulse labeled cells are not identical (Peterson et al., 1995; Wada et al., 1995). The two chaperones bind to major histocompatibility complex (MHC) class I antigens at different stages of maturation (Sadasivan et al., 1996; Van Leeuwen and Kearse, 1996), and in the case of influenza virus hemagglutinin (HA), calreticulin dissociates more rapidly than calnexin as folding proceeds (Hibert et al., 1996). It is possible that the two chaperones have distinct functions during glycoprotein biosynthesis.

In this paper, we have addressed the functional differences between calnexin and calreticulin using the well characterized influenza HA as a model substrate. After mutating the seven N-linked glycosylation consensus sequences in different combinations, we observed that effects on calnexin and calreticulin binding were distinct. Calreticulin binding depended on the glycans located in the more rapidly folding, globular top domain whereas calnexin appeared to be able to bind to glycans all over the molecule. Evidence was obtained for the presence of ternary HA–calnexin–calreticulin complexes. We also analyzed the role of individual oligosaccharides in the folding process, and found that some of them affected both rate and efficiency.

Materials and Methods

Reagents

Components for the cell-free translation, translocation, and folding system (rabbit reticulocyte lysate, amino acids, DTT, and RNasin) were purchased from Promega Corp. (Madison, WI). The canine pancreas microsomes were a generous gift of R. Gilmore (University of Massachusetts Medical Center, Worcester, MA). Radiolabeled [35S]methionine/cysteine, oxidized glutathione (GSSG), and CHAPS (3-
[330x584]C for cotranslational folding studies. After posttranslational folding, HA was translated in the presence of 4.0 mM GSSG at 32°C for cotranslational folding studies. The redox conditions were adjusted so that the translocated HA molecules would undergo disulfide bond formation and folding (Hibert et al., 1995, 1996). After synthesis, the microsomes were treated with NEM to alkylate-free sulfhydryl groups, and solubilized with nonionic detergent. After immunoprecipitivating with antibodies to HA, calnexin, or calreticulin, the samples were analyzed without reduction by SDS-PAGE and autoradiography.

Our previous studies have shown that the newly synthesized, nonreduced, full-length HA runs as three bands (Braakman et al., 1991; Marquardt et al., 1993; Braakman, I., and A. Helenius, manuscript in preparation). The band called IT1 corresponds to an ensemble of incompletely folded HA molecules that lack disulfide bonds C52–C277 and C14–C466. These two disulfide bonds form large loops in the HA molecule, and their presence considerably increases the electrophoretic mobility of the SDS complexes.
The IT2 band contains molecules that have disulfide C52–C277 but do not contain C14–C466. The fastest migrating band (NT, for native) is composed of monomeric molecules that have both of these disulfide bonds.

The immunoprecipitations in Fig. 1 (A–C, lanes 1–3) indicate that calreticulin bound preferentially (but not exclusively) to the less oxidized folding intermediates IT1 and IT2. In contrast, calnexin bound to all three forms, IT1, IT2, and NT. When quantified by gel scanning, the average IT1/IT2/NT binding ratio in 10 experiments was 1.0:0.65:0.63 for calnexin and 1.0:0.57:0.16 for calreticulin, with normalization with anti-HA precipitations. Since folding of HA proceeds from IT1 and IT2 to NT (Braakman et al., 1991; Marquardt et al., 1993), the results indicate that calreticulin participates mainly in early stages of folding whereas calnexin association occurs throughout the folding of HA monomers.

Differential binding of calnexin and calreticulin was also observed when HA folding occurred posttranslationally. In this case translation was performed in the presence of a high concentration of the reducing agent DTT, and folding began posttranslationally with the addition of GSSG (Fig. 1, lanes 4–9) (Marquardt et al., 1993). The presence of DTT not only inhibits formation of disulfide bonds in HA but also prevents efficient binding of calnexin and calreticulin (Fig. 1, lane 4; Hebert et al., 1995). When GSSG was added, calnexin and calreticulin associated with HA. The same preference of calreticulin for IT1 and IT2 was seen again (Fig. 1, lanes 5–9), indicating that it was determined by the intrinsic properties of the HA molecule. It did not depend on the vectorial translocation of the growing chain into the ER, or on limited accessibility of the HA polypeptide within the translocon complex.

To determine whether the differences in calnexin and calreticulin binding could be observed in live cells, we used a 2-D SDS-PAGE system recently developed in our lab to simultaneously monitor folding of nascent and full-length HA chains. CHO cells infected with influenza virus were pulse labeled for 1.5 min with $[^{35}S]$methionine/cysteine. Some experiments were performed in the presence of castanospermine, a competitive inhibitor of the ER α-glucosidases, to inhibit calnexin and calreticulin binding to HA (Hammond et al., 1994; Chen et al., 1995; Hebert et al., 1995; Peterson et al., 1995). The cells were alkylated, solubilized, and the lysates subjected to immunoprecipitation with antibodies against the NH$_2$-terminal peptide of HA, to calnexin or to calreticulin. To separate the chaperone-bound HA molecules from other substrates, the anti-calnexin and -calreticulin precipitates were dissolved and reprecipitated with the anti-HA antibodies. The samples were then subjected to 2-D SDS-PAGE in which the first dimension was done by the intrinsic properties of the HA molecule. It did not depend on the vectorial translocation of the growing chain into the ER, or on limited accessibility of the HA polypeptide within the translocon complex.

To determine whether the differences in calnexin and calreticulin binding could be observed in live cells, we used a 2-D SDS-PAGE system recently developed in our lab to simultaneously monitor folding of nascent and full-length HA chains. CHO cells infected with influenza virus were pulse labeled for 1.5 min with $[^{35}S]$methionine/cysteine. Some experiments were performed in the presence of castanospermine, a competitive inhibitor of the ER α-glucosidases, to inhibit calnexin and calreticulin binding to HA (Hammond et al., 1994; Chen et al., 1995; Hebert et al., 1995; Peterson et al., 1995). The cells were alkylated, solubilized, and the lysates subjected to immunoprecipitation with antibodies against the NH$_2$-terminal peptide of HA, to calnexin or to calreticulin. To separate the chaperone-bound HA molecules from other substrates, the anti-calnexin and -calreticulin precipitates were dissolved and reprecipitated with the anti-HA antibodies. The samples were then subjected to 2-D SDS-PAGE in which the first dimension was done by the intrinsic properties of the HA molecule. It did not depend on the vectorial translocation of the growing chain into the ER, or on limited accessibility of the HA polypeptide within the translocon complex.

The three spots at the extreme right of Fig. 2 A represent the labeled IT1, IT2, and NT forms of the full-length HA. The spurs on the left of the spots emanating diagonally correspond to the labeled nascent chains of variable length (Chen et al., 1995). The spur connected to the IT1 spot contains nascent chains that lack disulfide bonds C52–C277 and C14–C466. They may contain one or more of the small intramolecular disulfide loops. The spur connecting to the IT2 spot corresponds to nascent chains that had disulfide C52–C277 but not C14–C466.

It is apparent from the gel patterns in Fig. 2, A–C that both nascent and full-length HA molecules associate with calnexin and calreticulin in living cells. Association was dependent on the trimming of glucoses from the N-linked oligosaccharides because it was blocked by castanospermine (data not shown) (Hammond et al., 1994; Chen et al., 1995; Peterson et al., 1995). From the length of the main spur, it could be estimated that binding occurred by the time the length of the nascent chains was ~40 kD. Among the full-length forms, calreticulin bound preferentially to the least folded molecules (IT1) whereas calnexin bind to all forms (IT1, IT2, and NT).

Here for the first time, we found that the soluble and luminal calreticulin has access to the growing nascent chains in the ER of living cells. These results also confirmed that calreticulin participates mainly in the early stages of folding whereas calnexin is involved throughout. The capacity of the chaperones to bind to nascent chains indicated, moreover, that trimming of the two outermost glucoses from the N-linked glycans can occur on the growing nascent chain in <1 min after addition of the glycan by the oligosaccharyl transferase.
HA Forms Ternary Complexes with Calnexin and Calreticulin

The HA monomer has seven N-linked glycans. Four are located in the stem domain attached to asparagines 8, 22, 38, and 483. One is in the hinge region (Asn 285), and two (Asn 81 and Asn 165) are in the top domain (Fig. 9). With so many glycans distributed widely over the surface, the protein has the potential of binding more than one calnexin or calreticulin molecule at a time.

To test whether ternary complexes containing HA and both of the chaperones occurred in the ER-derived microsomes, sequential double immunoprecipitations were performed (Fig. 3, A and B). Primary precipitations with α-HA, -calnexin, or -calreticulin (Hebert et al., 1996) showed that 53% of the ^35S-labeled HA was associated with calnexin, and 45% with calreticulin (Fig. 3, lanes 3 and 6, respectively). When the supernatants from these precipitations were reprecipitated with the same antibodies, no additional HA was brought down (Fig. 3, lanes 2, 4, and 7), indicating that the primary precipitations were quantitative. In contrast, when aliquots of the supernatants were reprecipitated after the first precipitation with antibodies against the second chaperone, additional HA was precipitated (Fig. 3, lanes 5 and 8). This HA corresponded to molecules that were associated only with one of the chaperones. The relatively small amount of HA in these complexes suggested that most of the complexes contained both chaperones.

When the radioactivity in the various bands was quantified using a phosphorimager (Fig. 3 B), it was found that 10% of the HA was present in calreticulin–HA complexes and 8% in calnexin–HA complexes. As much as 40% of the HA occurred in ternary calnexin–calreticulin–HA complexes. A large fraction of the HA was thus associated with both chaperones simultaneously. Consistent with calreticulin’s preference for IT1 and IT2, the ternary complexes and the binary calreticulin–HA complexes were enriched in IT1 and IT2 (Fig. 3, lanes 6 and 5, respectively). The binary calnexin–HA complexes contained more HA of the fully oxidized NT type (Fig. 3, lane 8). It could be concluded that the majority of the chaperone-bound HA occurred in complexes containing both calnexin and calreticulin.

Maturation and Calnexin/Calreticulin Binding of Single Glycosylation Site Mutants

Since a large amount of HA was associated with both chaperones, it was of interest to determine whether these chaperones bound to different regions of the HA molecule. A series of mutants were constructed in which glycosylation sites were eliminated by point mutations in the consensus glycosylation sites. Typically, the Asn in the consensus sequence was replaced with Gln. In specific cases, other changes were also made to confirm that the changes were due to the loss of the oligosaccharide and not to the amino acid change.

In the first series of mutants, glycosylation sequences were eliminated one by one. The mutant proteins were translated in the presence of microsomes and analyzed for folding and oligomerization as well as calnexin/calreticulin binding. We found that six out of seven mutants were able to fold to the NT form (Fig. 4 A). The same mutant pro-
Proteins also assembled into trimers, judging by immunoprecipitation, with a trimer-specific anti-HA monoclonal antibody (Fig. 4B). Clearly, the oligosaccharide moieties in positions 8, 22, 38, 165, 285, and 483 were dispensable when eliminated singly.

However, the folding of some of these six mutant molecules was not identical to the wild type in every respect. For example, whereas the rate of NT formation for mutants Δ8, Δ22 and Δ38 was similar to wild type, only minor amounts of IT2 were seen as intermediates. Evidently, the transition from IT2 to NT (i.e., the formation of the disulfide C14–C466) was accelerated.

Deletion of the COOH-terminal stem glycan in Δ483, on the other hand, resulted in oxidation to NT at a rate faster than the wild type (Fig. 4A, below in Fig. 6B, and data not shown). Such differences indicated that although changes in these six glycosylation sites did not affect the final outcome of the folding process, they did affect the kinetics of folding and the expression of intermediate forms.

Mutant Δ81, which lacks one of the two oligosaccharides present in the top domain, was an exception. It was greatly inhibited in its ability to fold beyond a form that was either fully reduced or equivalent to IT1 with some of the small disulfide bonded loops in place (Fig. 4A). Surprisingly, this protein seemed unable to form interchain disulfide bonds which are common for misfolded forms of HA (Hurtley et al., 1989; Braakman et al., 1992b). Abolishing the glycosylation site by a T83A mutation rather than the N81Q mutation resulted in the same folding phenotype (data not shown) indicating that the effect was not due to a specific amino acid change. The inability of Δ81 to oxidize was likely due to disruption in the formation of disulfide C67–C76, which is very close to this glycan (see Fig. 9), and known to be crucial for formation of other intrachain disulfides in the HA monomer (Braakman, I., and A. Helenius, manuscript in preparation).

When the calnexin and calreticulin binding to the mutant proteins was measured by coimmunoprecipitation, no significant differences compared to wild-type HA were seen for any of them, including Δ81 (Fig. 4C). With exception of Δ483, they also bound normal levels of calreticulin. The 40% reduction in calreticulin binding observed for Δ483 was likely caused by the rapid folding of this mutant HA, resulting in a shorter exposure of the preferred cal-

---

**Figure 4.** Folding, oligomerization, and calnexin/calreticulin binding of single glycan deletion mutants. (A) Wild-type and single glycosylation mutants of HA were translated under oxidizing conditions in the presence of canine pancreas microsomes at 32°C for 1 h. At the indicated times, samples were removed, alkylated, lysed, and immunoprecipitated with anti-HA antibodies. HA was resolved by nonreducing SDS-PAGE and autoradiography. (B) Translations were performed as above with oxidation carried out for 8 h at 32°C. The alkylated HA was immunoprecipitated with anti-trimer specific HA antibodies and resolved by reducing SDS-PAGE and autoradiography. (C) Translation of 35S-labeled HA were performed as in A for 1 h at 32°C. After alkylation, HA was immunoprecipitated with anti-HA, -calnexin, and -calreticulin antibodies. HA was resolved upon nonreducing and reducing SDS-PAGE and autoradiography. The fraction HA coprecipitating with calnexin or calreticulin was calculated as the amount of anti-calnexin or -calreticulin precipitable HA divided by the total HA precipitated with anti-HA antibodies as described in Fig. 3.
Calreticulin binding conformers, IT1 and IT2 (see below). We concluded that no single glycan in HA served as the exclusive binding site for either calnexin or calreticulin.

**Calreticulin Binds Preferentially to Top and Hinge Glycans**

Since we were unable to disrupt binding of calnexin or calreticulin with single glycan deletions, combinations of multiple deletions were tested. Depending on the combination, differences in chaperone binding now emerged. Elimination of the three sites in the top/hinge domain (Δ81,165,285) resulted in almost complete loss of calreticulin binding (Fig. 5A). In contrast, the mutant protein bound almost normal amounts of calnexin. The top/hinge glycans were evidently needed for calreticulin but not for calnexin binding.

Surprisingly, the reverse experiment in which three stem glycans (Δ8,22,38) were eliminated also showed reduced binding of calreticulin (Fig. 5A), whereas calnexin binding was only marginally reduced. Further studies showed that the apparent inability of calreticulin to bind to this particular mutant was caused by its accelerated folding (see below). The early oxidative intermediates IT1 and IT2 that normally bind calreticulin were so short-lived in this mutant that the calreticulin-binding phase during folding could not be experimentally observed.

To correct for this effect, an additional mutation (C305S) was introduced. This mutation prevents the formation of disulfide bond C281−C305 which is needed to convert IT1 and IT2 to NT (Braakman, I., and A. Helenius, manuscript in preparation). It effectively traps the protein in a form equivalent to early folding intermediates. With this mutation in place, the protein stayed as IT1 (data not shown). In this mutant background, the loss of the three-stem domain glycans in positions 8, 22, and 38 had little or no effect on calreticulin binding (Fig. 5B). However, removal of the three top/hinge domain glycans resulted in a 64% drop, confirming the preferential binding of calreticulin to the top/hinge glycans. Removal of all three glycans was required for calreticulin binding to be affected. Calnexin binding was again virtually unaffected by the loss of glycans, suggesting that it can bind both to top/hinge and the stem domain glycans.

**Calnexin and Calreticulin Binding to HA Mutants in Living Cells**

To test whether elimination of multiple glycans would have similar effects on calreticulin and calnexin binding in live cells, wild-type HA and two mutants were expressed in CHO cells by use of the T7-based vaccinia expression system. 4 h after infection, cells were pulsed with [35S]methionine/cysteine for 2 min at 37°C. Alkylated and lysed samples were then processed as above.

binding of calreticulin to Δ8,22,38/C305S, but its binding to Δ81,165,285/C305S was found to be dramatically decreased. Taken together, the results suggest that in live cells, as well as in microsomes, calreticulin binds preferentially, but not exclusively, to the top domain glycans. Calnexin, on the other hand, can use glycans in any part of the protein. Since the stem disulfides are the last to form during folding (Braakman et al., 1991), the results are consistent with the observation that calnexin binding continues for a longer period during HA folding than calreticulin binding (Fig. 1).

**Calnexin and Calreticulin Binding to Mutants with Decreasing Numbers of Oligosaccharide Moieties**

That calnexin can bind to many parts of the HA molecule was confirmed by a series of mutants in which glycans were progressively removed one at a time from the NH2 terminus towards the COOH terminus (Fig. 6, lanes 2–8), or conversely, from the COOH terminus to the NH2 terminus (Fig. 6, lanes 9–15). The stepwise loss of glycans resulted

Figure 5. Mapping of calnexin and calreticulin binding sites by mutating consensus glycosylation sequences. The consensus sequences for glycosylation were eliminated by changing the Asn to Gln. The HA was translated under oxidizing conditions for 1 h at 32°C. (A) The fraction of total HA coprecipitating with the various antisera is plotted. (B) Binding to calnexin and calreticulin was monitored as described above for a set of HA mutants which had, in addition to mutations in the designated consensus glycosylation sites, an additional mutation, C305S, which arrested HA oxidation in the IT1 conformation. The disruption of disulfide 281–305 by changing Cys305 to a Ser results in a 38S-HA was expressed in CHO cells by use of the T7-based vaccinia expression system. 4 h after infection, cells were pulsed with [35S]methionine/cysteine for 2 min at 37°C. Alkylated and lysed samples were then processed as above.
in a ladder of HA molecules with faster electrophoretic mobilities the fewer the oligosaccharides. Thus, with about in a ladder of HA molecules with faster electrophoretic mobility of the untranslocated HA (denoted 2-kD steps for each glycan removed, the HA bands approximated when the three glycans in the NH2-terminal part of the HA folding and the formation of NT. Removal of some of these glycans, or inhibition of calnexin binding to them, resulted in faster folding. The accelerated folding has been previously observed in microsomes when calnexin and calreticulin binding is inhibited with castanospermine (Hebert et al., 1996). Taken together with the observed acceleration of folding of mutants Δ8,22, Δ8,22,38, Δ483 and Δ285,483 in microsomes (Figs. 4 A and 6 B), the data suggest that calnexin binding to the oligosaccharides present in the stem domain in positions 8, 22, 38, and 483 serves to slow down HA folding and the formation of NT. Removal of some of these glycans, or inhibition of calnexin binding to them, results in faster folding.

As expected, the Δ81 mutant folded inefficiently to the NT form in the live cells (Fig. 7 C). Essentially no protein reached the IT2 or NT forms after a 2-min pulse. A similar result was obtained whether N81Q mutation or the T83A mutation was used to eliminate the consensus glycosylation sequence (data not shown). The folding defect ob-
lysed and precipitated with antibodies raised against a peptide methionine for 2 min. Immediately after labeling, the cells were assayed in living cells, cotranslational folding of the HA was analyzed, and assayed for folding and calnexin and calreticulin binding. The anchor− HA was found to undergo normal folding and calnexin preipitated 82% of the wild type, but only 54% of anchor− HA (Fig. 8). The soluble HA thus associated somewhat less efficiently with the membrane-bound chaperone. Presumably, the oligosaccharides in the stem region of the anchor− HA were not as easily accessed by calnexin as in the membrane-bound wild-type HA.

**Discussion**

Our analysis of wild-type and mutant forms of HA confirmed the importance of the N-linked oligosaccharides in the general process of glycoprotein folding calnexin and calreticulin binding. Not only is the presence of N-linked glycans necessary for HA folding as previously shown (Hurtley et al., 1989; Gallagher et al., 1992), but their number and location in the molecule are also crucial. The results showed that the glycans affect the structural maturaion of the protein locally and globally. They influence the rate of folding and the formation of disulfide bonds. By determining the interactions with chaperones such as calnexin and calreticulin, they help to define the order progression of folding from one domain to the next. Calnexin and calreticulin, although working according to the same overall principles, were found to display distinct interactions with folding intermediates of HA.

When growing nascent chains of wild-type HA were analyzed, it could be shown that both calnexin and calreticulin already begin to associate during translation and translocation. Association was first detected in cells when the HA chain was about half finished, i.e., at a time when it was still ~1 min from termination (Braakman et al., 1991). At this time, five of the seven oligosaccharide chains (N8, 22, 38, and 81) had been added. Since calnexin and calreticulin binding was castanospermine sensitive, at least some of them must have been trimmed to the monoglucosylated form by glucosidase I and II. It is known that these ER glucosidases have access to nascent chains, and that glucose trimming is rapidly initiated (Hubbard and Ivatt, 1981). No indication of disulfide bond formation was detectable at the time of initial calnexin and calreticulin association.

Removal of NH2-terminal glycans in positions N8, 22, and 38 resulted in HA molecules that reached the fully oxidized form more rapidly than wild-type HA. When assayed in living cells, cotranslational folding of the Δ8, 22, and 38 mutant protein was also found to fold faster. We have previously reported that inhibition of calnexin and calreticulin binding by addition of castanospermine has a similar effect; the rate of NT formation is accelerated and aberrant nonnative disulfide bonds are formed cotranslationally (Chen et al., 1995; Hebert et al., 1996). Based on these observations, we hypothesized that by binding to the NH2-terminal glycans, the chaperones sequester the NH2-terminal portion of the growing chain. This is likely to be important because the sequence comprising residues 1–50...
The preferential binding to these glycans may have several implications. First, the oligosaccharides in the top part of the molecule may be more easily accessed from the lumen of the ER, thus attracting the soluble calreticulin more efficiently than the membrane-bound calnexin. Second, being a membrane protein, calnexin may bind more efficiently to the membrane-proximal stem domain glycans, with the result that only the top domain glycans are left for calreticulin to bind to. Support for this notion comes from the observed reduction in calnexin binding to anchor-free HA compared to wild-type HA. Mere membrane binding that affects substrate specificity is shown by the observations of Wada and co-workers (Wada et al., 1995), who found that the pattern of calreticulin-bound proteins in HepG2 cells became similar to that of calnexin when the calreticulin was anchored to the membrane by calnexin’s transmembrane region.

It is also possible that the steric arrangement of the top domain glycans may find a better fit in calreticulin’s binding site. The glycan binding site(s) are thought to be located in the P-domains of calnexin and calreticulin. Although highly homologous to calnexin’s, the P-domain of calreticulin is smaller and has three double sets of sequence repeats instead of four (Wada et al., 1991; Michalak et al., 1992; David et al., 1993). These differences in the proteins may contribute to selectivity in binding.

Finally, the distinct binding specificities could be determined by protein–protein contacts. Such interactions have been postulated to occur between MHC class I and II heavy chains and calnexin (Arunachalam and Cresswell, 1995; Williams, 1995; Zhang et al., 1995). Although protein–protein contacts are not required for the formation of stable complexes between substrate glycoproteins and the two chaperones (Rodan et al., 1996; Zapun et al., 1997), they cannot be ruled out as additional factors contributing to the specificity of binding.

As a consequence of binding to the top/hinge glycans, calreticulin dissociates sooner from the folding HA chain than calnexin. This is consistent with results indicating that folding of HA begins from the top domain and proceeds from there towards the stem (Braakman et al., 1992; David et al., 1993). These differences in the proteins may contribute to selectivity in binding.

The progression of folding is best illustrated by the orderly, hierarchical formation of disulfide bonds that starts with the formation of either C67–C76 and C97–C139 in the top domain, and proceeds to the hinge domain disulfide C281–C305, then disulfide C52–C277 (Fig. 9) (Braakman, I., and A. Helenius, manuscript in preparation). We have, in this study, taken advantage of this stringent oxidation program by using the mutation C305S, which arrests oxidation at IT1, to study calreticulin binding to glycosylation mutants that would otherwise fold too rapidly.

One possible reason why calreticulin dissociates before the HA molecule is fully folded is that the top domain fails to be reglucosylated by the UDP-glucose:glycoprotein glucosyltransferase. It may no longer be recognized as unfolded by this folding sensor. This would imply that the transferase senses unfold locally rather than globally. In other words, in a protein with several domains, only the misfolded ones may serve as substrates. Further studies are needed to define how close to a misfolded domain a glycan has to be to serve as a substrate for reglucosylation.

The elimination of most of the glycans singly did not affect the outcome of the folding process. It did, however, affect the folding rate by either increasing (glycans 8, 22, 38, and 483) or decreasing it (glycans 81, 165, and 285). Of the seven oligosaccharides only glycan N81 proved to be essential for HA folding in microsomes. Mutant HA molecules devoid of this glycan were virtually incapable of acquiring intrachain disulfide bonds. They also failed to form the interchain disulfides typically observed when HA misfolds (Hurtley et al., 1989; Marquardt and Helenius, 1992).

Our results indicate that as the folding of the HA chain progresses, calreticulin associated preferentially with the first 38 residues of the X31 HA used in this study. Cys 14 emerges from the translocon in cells 1.5 min or more before its partner, Cys 466. By binding to the NH2-terminal glycans, the chaperones may serve to postpone the folding of the NH2-terminal segment and prevent Cys 14 from oxidizing with the eight intervening cysteines in the polypeptide chain. This could, in fact, explain why three glycans are located in the first 38 residues of the X31 HA used in this study.

The preferential binding to these glycans may have several reasons. First, the oligosaccharides in the top part of the molecule may be more easily accessed from the lumen of the ER, thus attracting the soluble calreticulin more efficiently than the membrane-bound calnexin. Second, being a membrane protein, calnexin may bind more efficiently to the membrane-proximal stem domain glycans, with the result that only the top domain glycans are left for calreticulin to bind to. Support for this notion comes from the observed reduction in calnexin binding to anchor-free HA.

![Image](50x418 to 230x750)

Figure 9. Calnexin and calreticulin binding regions on HA. The structure of the ectodomain of HA, modified from Wiley and co-workers (Wilson et al., 1981) is depicted with N-linked glycans and the disulfide bonds designated by the circles and the ball-and-sticks, respectively. The large disulfide loop, Cys 14–466, is represented by the unfilled ball-and-sticks. The large disulfide loop, Cys 14–466, is the Asn residue that contain the N-linked glycosylations. The progression of folding is best illustrated by the orderly, hierarchical formation of disulfide bonds that starts with the formation of either C67–C76 and C97–C139 in the top domain, and proceeds to the hinge domain disulfide C281–C305, then disulfide C52–C277 (Fig. 9) (Braakman, I., and A. Helenius, manuscript in preparation). We have, in this study, taken advantage of this stringent oxidation program by using the mutation C305S, which arrests oxidation at IT1, to study calreticulin binding to glycosylation mutants that would otherwise fold too rapidly.
The protein remained essentially unoxidized for a long time. The explanation for the poor folding may be the close vicinity of N81 to a top domain disulfide bond, C67–C76 (Fig. 9). Interestingly, when the cysteines of this disulfide are mutated to serines a similar oxidation-deficient phenotype is observed (Braakman, I., and A. Helenius, manuscript in preparation). Thus, the most likely explanation for the folding defect in Δ81 is that the formation of a disulfide bond C67–C76 is inhibited when the glycan is missing. This disulfide is important as it serves as an early obligatory link in a chain of downstream oxidation events.

It is somewhat surprising that glycan N81 should prove to be so essential because it is not conserved among influenza strains. The conserved glycans are all in the stem domain and correspond to N22, 38, and 483 in the X31 HA. However, Gallagher and co-workers (1992) have suggested that the oligosaccharides in positions 8 or 81 of the X31 HA may, in fact, be essential for maturation. They found that the HA of the Japan strain, which does not have these glycans, reaches a transport-competent conformation after tunicamycin treatment, whereas the HA of X31 HA that has them is completely tunicamycin sensitive (Gallagher et al., 1992). However, when they analyzed single-site glycosylation mutants of X31 HA, they found that no single glycan was essential for the acquisition of a transport-competent conformation in live cells. In their study, transport ability was analyzed after extended times of chase, whereas here we have monitored the folding or oxidation process. In live cells the Δ81 mutant displayed a clear-cut cotranslational folding defect, but after a time some of it was able to reach a folded conformation (data not shown) and therefore, would be expected to be competent for transport.

The differences observed among strains of influenza are further illustrated by studies performed by Roberts and co-workers, who have found that two out of three of the conserved stem domain glycans are required for the proper maturation and transport of HA in the strain of fowl plague virus (A/FPV/Rostock/34[H7N1]) (Roberts et al., 1993). Here, the location of the top glycans in this strain are very different from that of the X31 strain glycans. Furthermore, most HAs from avian and porcine influenza strains have no glycans in the top or hinge domain. Depending on the strain, glycans can, therefore, be dispensable in the formation of the top domain disulfide bonds.

In summary, we find that N-linked glycosylation on HA influences the folding process in many complex ways. One thing is clear, however; the N-linked glycans allow the newly synthesized protein to interact with the calnexin–calreticulin cycle. Though not totally essential for the folding of HA, this serves to decrease the rate of folding, prevent premature trimORIZATION, inhibit degradation, increase the efficiency of folding, and expose the protein to efficient quality control (Hammond et al., 1994; Hebert et al., 1996). HA folding involves calnexin and calreticulin in an elaborate series of interactions starting on the nascent chain. Binding of calnexin and calreticulin to distinct oligosaccharides and different domains is apparently used by HA to organize the processes of folding. For example, it seems likely that calnexin and calreticulin are used to temporarily sequester the NH2-terminal segment of HA, thus promoting more efficient cotranslational folding of NH2-terminal and COOH-terminal sequences to form the stem domain.

It is also clear that glycans have effects on HA folding that are independent of calnexin and calreticulin. For the X31 HA, this is illustrated by the observation that the protein is completely misfolded in the presence of tunicamycin but only partially affected by castanospermine (Hurtley et al., 1989; Hammond et al., 1994; Hebert et al., 1996). The requirement for a glycan in position N81 provides one example of a direct effect. The importance of this oligosaccharide seems to be based on a local influence on the formation of a nearby disulfide bond. In addition to local effects, the presence of oligosaccharides is likely to have more global influences on folding by increasing overall polarity of folding intermediates, thus counteracting their tendency to aggregate (Kern et al., 1992; Marquardt and Helenius, 1992; Kern et al., 1993).

We would like to acknowledge R. Gilmore (University of Massachusetts Medical Center), I. Braakman (University of Amsterdam, Amsterdam, The Netherlands), and T. Marquardt (University of Muenster, Muenster, Germany) for providing the canine pancreas microsomes and assistance with some of the glycosylation and truncation mutants, respectively. We would also like to thank N. Ayad (Yale University, New Haven, CT) and E.S. Trombetta (Yale University) for helpful discussions.

This work was supported by the Patrick and Catherine Weldon Donaghy Medical Research Foundation to D.N. Hebert, and by National Institutes of Health grants to A. Helenius (5-RO1-GM38346 and 1-RO1-GM52972) and J.-X. Zhang (1-532-DKO9309).

Received for publication 31 July 1997 and in revised form 28 August 1997.
Kern, W.-J., P.H. Cameron, D.Y. Thomas, and J.J.M. Bergeron. 1993. Association of differently glycosylated variants of invertase from Saccharomyces cerevisiae. Protein Sci. 2:1862–1868.

Marquardt, T., and A. Helenius. 1992. Misfolding and aggregation of newly synthesized proteins in the endoplasmic reticulum. J. Cell Biol. 117:505–513.

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

Michalak, M., R.E. Milner, K. Burns, and M. Opas. 1992. Calreticulin. Biochem. J. 285:681–692.

Ou, W.-J., P.H. Cameron, D.Y. Thomas, and J.J.M. Bergeron. 1993. Association of folding intermediates of glycoproteins with calnexin during protein maturation. Nature (Lond.). 364:771–776.

Peterson, J.R., A. Ora, P. Nguyen Van, and A. Helenius. 1995. Transient, lectin-like association of calreticulin with folding intermediates of cellular and viral glycoproteins. Mol. Biol. Cell. 6:1173–1184.

Rajagopalan, S., and M.B. Bremer. 1994. Calnexin retains unassembled major histocompatibility complex class I free heavy chains in the endoplasmic reticulum. J. Exp. Med. 180:407–412.

Roberts, P.C., W. Garten, and H.-D. Klönk. 1993. Role of conserved glycosylation sites in maturation and transport of influenza A virus hemagglutinin. J. Virol. 67:3048–3060.

Rodan, A.R., J.F. Simons, E.S. Trombetta, and A. Helenius. 1996. N-linked oligosaccharides are necessary and sufficient for association of RNase B with calnexin and calreticulin. EMBO (Eur. Mol. Biol. Organ.) J. 15:6921–6930.

Sadasivan, B., P.J. Lehner, B. Ortmann, T. Spies, and P. Cresswell. 1996. Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. Immunity. 5:103–114.

Sambrook, J., E.F. Fritsch, and T.maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.

Singh, I., R.W. Doms, K.R. Wagner, and A. Helenius. 1990. Intracellular transport of soluble and membrane-bound glycoproteins: folding, assembly, and secretion of anchor-free influenza hemagglutinin. EMBO (Eur. Mol. Biol. Organ.) J. 9:631–639.

Sousa, M.C., M.A. Ferrero-Garcia, and A.J. Parodi. 1992. Recognition of the oligosaccharide and protein moieties of glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase. Biochemistry. 31:97–105.

Spiro, R.G., Q. Zhu, V. Bhoyroo, and H.-D. Söling. 1996. Definition of the lectin-like properties of the molecular chaperone, calreticulin, and demonstration of its copurification with endomannosidase from rat liver Golgi. J. Biol. Chem. 271:11588–11594.

Suh, P., J.E. Bergmann, and C.A. Gabriel. 1989. Selective retention of monoglucosylated high mannose oligosaccharides by a class of mutant vesicular stomatitis virus G proteins. J. Cell Biol. 108:811–819.

Tector, M., and R.D. Salter. 1995. Calnexin influences folding of human class I histocompatibility proteins but not their assembly with β2-microglobulin. J. Biol. Chem. 270:19638–19642.

Trombetta, S.E., and A.J. Parodi. 1992. Purification to apparent homogeneity and partial characterization of rat liver UDP-glucose:glycoprotein glucosyltransferase. J. Biol. Chem. 267:9236–9240.

Van Leeuwen, J.E.M., and K.P. Kearse. 1996. Deglucosylation of N-linked glycans is an important step in the dissociation of calreticulin-class I-TAP complexes. Proc. Natl. Acad. Sci. USA. 93:13997–14001.

Van Leeuwen, J.E.M., and K.P. Kearse. 1997. Reglucosylation of N-linked glycans is critical for calnexin assembly with T cell receptor (TCR) α proteins but not TCR β proteins. J. Biol. Chem. 272:4179–4186.

Vassilakos, A., M.F. Cohen-Doyle, P.A. Peterson, M.R. Jackson, and D.B. Williams. 1996. The molecular chaperone calnexin facilitates folding and assembly of class I histocompatibility molecules. EMBO (Eur. Mol. Biol. Organ.) J. 15:1495–1506.

Wada, I., D. Rindress, P.H. Cameron, W.-J. Ou, J.J. Doherty II, D. Louvard, A.W. Bell, D. Dignard, D.Y. Thomas, and J.J.M. Bergeron. 1991. SSRs and associated calnexin are major calcium binding proteins of the endoplasmic reticulum membrane. J. Biol. Chem. 266:19599–19610.

Wada, I., S.-I. Imai, M. Kai, F. Sakane, and H. Kanoh. 1995. Chaperone function of calreticulin when expressed in the endoplasmic reticulum as the membrane-anchored and soluble forms. J. Biol. Chem. 270:20298–20304.

Ware, F.E., A. Vassilakos, P.A. Peterson, M.R. Jackson, M.A. Lehrman, and D.B. Williams. 1995. The molecular chaperone calnexin binds Glc4Man3GlcNAc2 oligosaccharides as an initial step in recognizing unfolded glycoproteins. J. Biol. Chem. 270:4697–4704.

Williams, D.B. 1995. Calnexin: a molecular chaperone with a taste for carbohydrate. Biochem. Cell. Biol. 73:123–132.

Wilson, I.A., J.J. Skehel, and D.C. Wiley. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution. Nature (Lond.). 289:366–373.

Zapun, A., S.M. Petrescu, P.M. Rudd, R.A. Dwek, D.Y. Thomas, and J.J.M. Bergeron. 1997. Conformation independent binding of monoglucosylated ribonuclease B to calnexin. Cell. 88:29–38.

Zhang, Q., M. Tector, and R.D. Salter. 1995. Calnexin recognizes carbohydrate and protein determinants of class I major histocompatibility complex molecules. J. Biol. Chem. 270:3944–3948.