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Folding of the human immunodeficiency virus type 1 envelope glycoprotein in the endoplasmic reticulum

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Abstract — The lumen of the endoplasmic reticulum (ER) provides a unique folding environment that is distinct from other organelles supporting protein folding. The relatively oxidizing milieu allows the formation of disulfide bonds. N-linked oligosaccharides that are attached during synthesis play multiple roles in the folding process of glycoproteins. They stabilize folded domains and increase protein solubility, which prevents aggregation of folding intermediates. Glycans mediate the interaction of newly synthesized glycoproteins with some resident ER folding factors, such as calnexin and calreticulin. Here we present an overview of the present knowledge on the folding process of the heavily glycosylated human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein in the ER. © 2001 Société française de biochimie et biologie moléculaire / Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

HIV-1 / endoplasmic reticulum / envelope glycoprotein

1. Introduction

The human immunodeficiency virus (HIV) type 1 is the infectious agent that causes acquired immunodeficiency syndrome (AIDS) [1]. Worldwide attempts are being made to develop an HIV vaccine directed against the envelope glycoprotein gp160. Gp160 is a type 1 integral membrane protein that plays a critical role in virus infection [2]. It consists of two non-covalently associated subunits, the soluble gp120, and the transmembrane domain containing subunit gp41. The envelope glycoprotein is the single HIV component required for fusion between the surface membranes of the virion and the target cell. The gp120 moiety binds to the CD4 receptor (primary receptor) and a specific chemokine receptor (the co-receptor, e.g., CCR5 or CXCR4) on the target cell [3]. Gp41 serves to anchor the oligomeric glycoprotein complex in the viral membrane and to mediate cell fusion [2]. Gp160 is the major target for the host's immune system and most of the neutralizing antibody activity in the sera of HIV-1 infected humans is directed against gp160 [4].

A wealth of information is available on the antigenic structure of native gp120 as expressed on the virus particle. Moore and co-workers showed which domains are located on the inside and outside of the folded gp120 structure [5, 6]. A large fragment of gp120 was crystallized [7] from which the variable loops and some other sequences had been deleted. For gp41, the complete ectodomain was crystallized and its structure determined [8].

Gp160 is co-translationally translocated into the ER. As soon as the nascent chain enters the ER lumen, it starts to fold, disulfide bonds are formed and the protein becomes glycosylated. Gp160 contains ~30 potential N-linked glycosylation sites, most of which are used (*figure 1*) [9]. Amongst the many different strains of the virus, the number and also the location of most sites is fairly conserved. Chaperones and folding enzymes, such as BiP, calnexin, calreticulin, and PDI, assist the folding of gp160 and isolated gp120 [10, 11] (and our unpublished results). Cleavage of the signal peptide occurs completely post-translationally [12]. When gp160 attains its native conformation and has trimerized, it is transported to the Golgi complex, where the protein is cleaved into gp120 and gp41 [13, 14]. Finally, the gp160 glycoprotein is transported to the plasma membrane where it is incorporated into the virion. The gp120 moiety is released to some extent into the cell culture medium as a monomer.

2. Endoplasmic reticulum

All proteins destined for transport through the secretory pathway start their journey by crossing the ER membrane.

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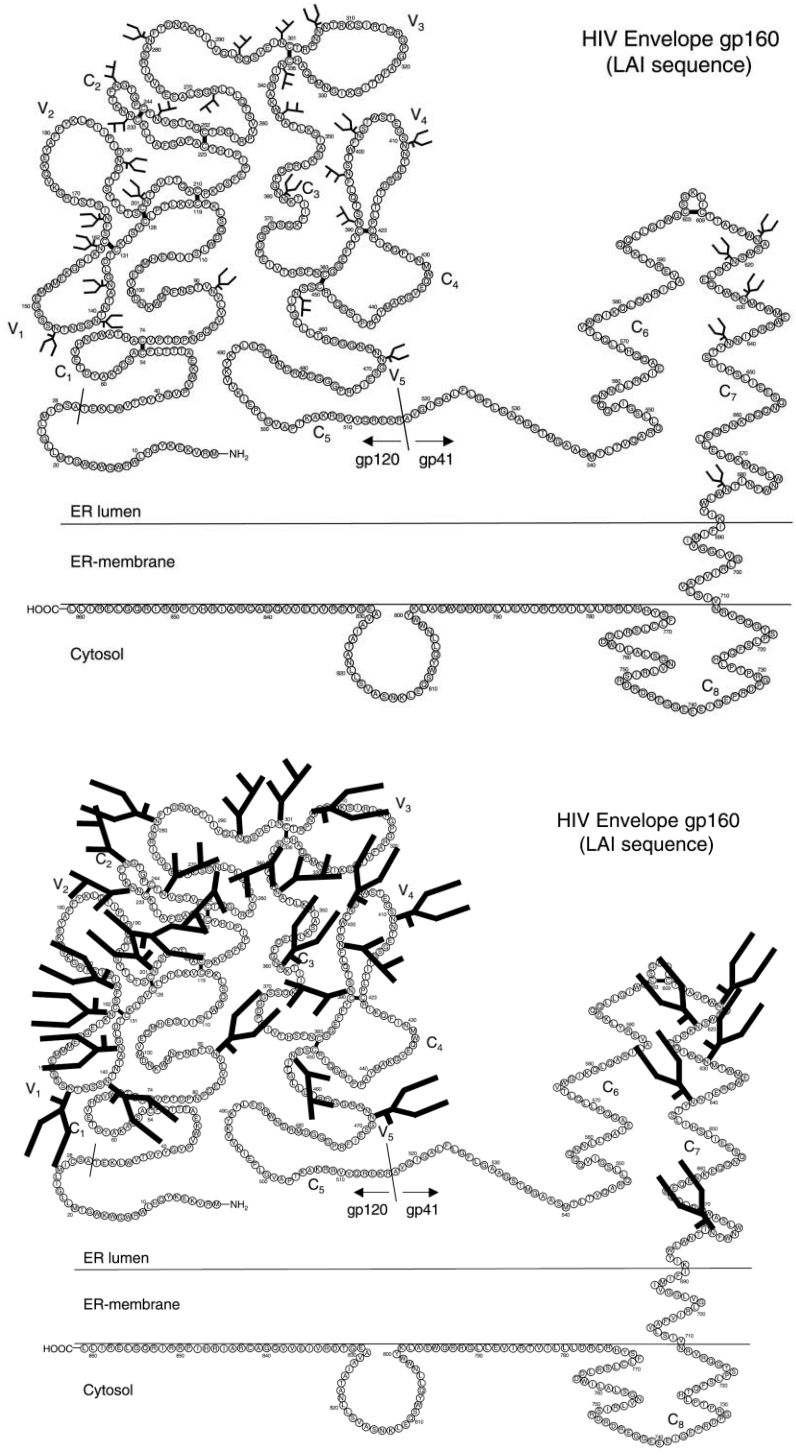

Figure 1. Schematic representation of HIV-1 LAI gp160. **A.** Disulfide bonds are shown as bars in the protein. Glycosylation sites containing high mannose-type or hybrid-type oligosaccharide structures as well as glycosylation sites containing complex-type oligosaccharide structures are indicated as antennae. **B.** The same picture, but now carbohydrate structures are drawn to scale. Modified by Eelco van Anken from Leonard et al. [9] and Ratner [62].

Depending on the signal the proteins carry, they are retained in the ER or transported to the Golgi complex and beyond. The ER lumen is distinct from other folding compartments: it's relatively oxidizing milieu supports disulfide bond formation and proteins can be glycosylated during synthesis.

Immediately after the N-terminus is translocated into the ER-lumen, glycosylation, disulfide bond formation and folding start. The N-terminus can reach its native secondary and tertiary structure even before translation of the C-terminus is completed. ER resident proteins, the so-called molecular chaperones, associate transiently during the process. Chaperones are needed to stabilize unfolded polypeptides, preventing inappropriate intra- and interchain interactions from occurring. They thereby prevent aggregation and facilitate the rate-limiting steps of folding [15].

By associating with newly synthesized unfolded or misfolded proteins, chaperones retain their substrates in the ER. Together with a check on conformational quality by other proteins, this constitutes the ER quality control system: proteins must be correctly and completely folded before being allowed to leave the ER for further transport throughout the cell. Parts of the quality control apply to all proteins whereas others are specific for selected proteins and protein families. The ER quality control eventually is responsible for retention in the ER, retrieval from downstream organelles, and ER-associated degradation (ERAD) [16].

3. The signal peptide

Proteins that are folded in the ER contain a specific signal that directs the protein to the ER membrane, the signal peptide or a signal-anchor sequence. Signal sequences usually are N-terminal extensions directing nascent proteins to the membrane of the ER. After translocation into the ER lumen, but before termination of the growing nascent chain, the signal sequence is removed by membrane attached ER signal peptidases [17].

One of the two known exceptions to the rule of co-translational signal peptide removal is HIV gp160: its signal peptide is cleaved completely post-translationally [18] (and our unpublished results). After synthesis is completed, gp160 remains N-terminally attached to the membrane via its signal peptide for at least 15 more min [12]. The signal peptide contains five positively charged amino acids. Substitution of these residues with neutral amino acid residues improved signal peptide removal in the isolated gp120 subunit, resulting in increased secretion of gp120 [19]. Li and co-workers showed in addition that replacement of the natural signal peptide with that of mellitin or interleukin-6 accelerated secretion [18]. The signal sequence of gp160, and especially its five positively charged amino acids, apparently plays a crucial role in determining the efficiency of signal peptide removal and hence protein secretion.

Inefficient cleavage of the signal peptide in general may interfere with proper folding of the target proteins or may lead to aggregation and misfolding [20]. Li and co-workers showed that late cleavage of the signal peptide retarded folding of the isolated gp120 subunit [12]. Our own data show that gp160 needs to fold to some extent before cleavage of the signal peptide occurs. Hence, a mutual relationship exists between gp160 folding and signal peptide removal: lack of removal delays or disturbs folding, and folding determines cleavage. Differences exist among the various HIV-1 strains in both their rates of folding and their timing of signal peptide cleavage, confirming their interdependence. The mutual dependence of folding and cleavage may help regulate the proper outcome of the folding process.

4. Disulfide bond formation

Many proteins that fold in the ER contain cysteines. The relatively oxidizing milieu in the ER lumen promotes their conversion to disulfide bonds, which can stabilize the three-dimensional structure of the protein during and/or after folding. Mutagenesis of cysteines often leads to retention of the protein in the ER and subsequent degradation by the proteasome [21]. For a long time, glutathione was thought to be responsible for the relatively oxidizing environment in the ER-lumen. In the cytosol, the ratio between reduced glutathione (GSH) and oxidized glutathione (GSSG) is ~60:1, whereas in the ER the ratio is ~3:1 [22]. The recent discovery of Ero1p in yeast and two homologous proteins, Ero1-Lα and β, in higher eukaryotes changed the view on glutathione as the primary redox buffer in the ER [23]. Ero1p supplies disulfide bonds to the target protein via protein disulfide isomerase (PDI) [24] and perhaps other PDI homologues. As a result, electrons flow from the newly synthesized protein via PDI to Ero1.

The HIV-1 envelope glycoprotein uses the cellular folding machinery present in the ER to fold into its biologically active conformation. Gp160 has 10 disulfide bonds; nine are located in gp120 and one in gp41. Disulfide bond formation of gp160 starts on the nascent chain and proceeds post-translationally [10]. The first molecules with native conformation can be detected around 10 min after synthesis but ~50% of the gp160 molecules reaches their native conformation more than 1 h later (*figure 2*). A large fraction of the folding intermediates needs up to days after synthesis to complete their folding process.

When gp160 is completely folded it becomes competent for CD4 binding [25]. This already happens in the ER since gp160-CD4 association leads to retention of CD4, and its subsequent downregulation of CD4 from the cell

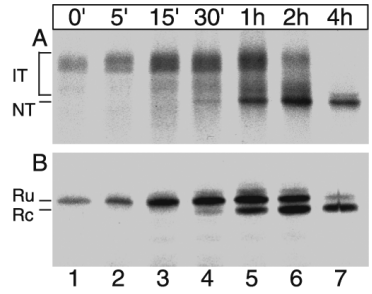

Figure 2. Folding assay for the isolated soluble subunit gp120 of HIV-1 LAI. HeLa cells were infected with vaccinia virus expressing the soluble gp120. Cells were pulse labeled for 2 min and chased for the indicated times. Detergent cell lysates were immunoprecipitated with polyclonal antibody recognizing all forms of gp120 and, after deglycosylation by endoglycosidase H, were subjected to non-reducing (**A**) and reducing (**B**) 7.5% SDS-PAGE. IT, folding intermediates; NT, native, completely oxidized form of gp120 from which the signal peptide has been removed; Ru, reduced uncleaved (signal peptide still attached) gp120; Rc, reduced and cleaved gp120. Electrophoretic mobility in the non-reducing gel represents compactness of the protein. Folding intermediates are less compact than the native form because they lack at least some disulfide bonds. Electrophoretic mobility in the reducing gel represents relative molecular mass. Removal of the signal sequence that targeted the protein to the ER causes an increase in mobility.

surface [26]. The capacity to bind CD4 is used as a measure for correctly folded gp160. Earl and co-workers determined that the fastest folding gp120 molecules can start to bind CD4 after 15 min [10], which agrees with the timing of appearance of the first CD4 binding site epitopes. These epitopes are present only on the native conformation, indicating that folding of the gp160 monomer should be virtually completed before it acquires CD4 binding capacity.

After gp160 is folded properly, it forms trimers and is transported to the Golgi complex. Formation of oligomers in gp160 from the HIV IIIB strain occurs with a half time of 30 min [10]. In our assay, HIV LAI gp160 oligomers were transported to the Golgi complex and cleaved into their subunits at such a high rate that oligomeric intermediates were too transiently present to detect. The folding kinetics of gp160 derived from various HIV-1 strains differs remarkably (our unpublished data). The IIIB envelope protein matures faster than LAI envelope, although they differ by only 25 residues. This would explain the differences found in folding kinetics and oligomer formation of gp160 by different groups. In the various cell lines tested, however, the pattern of folding intermediates and folding kinetics were similar. On the other hand, cleavage of gp160 into gp120 and gp41, an event that occurs late in the Golgi complex, was dependent on the cell line used [10], suggesting that a large heterogeneity existed in the detectable events during transport of gp160 and gp120 through the secretory pathway.

5. Protein disulfide isomerases

Various pieces of evidence suggest that non-native disulfide bonds are formed in gp160 during its folding process. A likely candidate to participate in the process of disulfide bond shuffling is PDI [27]. PDI is a multifunctional member of the thioredoxin superfamily with a molecular mass of 57 kDa, displaying both disulfide isomerase and chaperone activity. It is one of the most abundant soluble proteins in the lumen of the ER.

The two thioredoxin-like (CGHC) sequences of PDI have been characterized as the active sites for its isomerase activity [28]. In vitro experiments have shown that PDI can accelerate oxidation and can unscramble inappropriate disulfide bonds [27]. During the enzyme reaction, the oxidoreductase forms mixed disulfide bonds with its substrate. This covalent complex is an intermediate in the generation of native disulfide bonds within the substrate protein [29]. Helenius and co-workers showed recently that PDI and ERp57, another member of the PDI family, both are involved in disulfide oxidation and isomerization in living cells [30]. ERp57 forms a complex with the molecular chaperones calnexin and calreticulin (see below) and probably is specialized in the introduction of disulfide bonds into nascent glycoproteins.

PDI is also a well-characterized chaperone. In vitro experiments have shown that PDI increases the reactivation yield of the denatured non-disulfide bonded proteins D-glyceraldehyde-3-fosfate dehydrogenase (GAPDH) and rhodanese [31]. PDI is thought to bind unfolded proteins, and to thereby prevent aggregation. The chaperone activity of PDI seems to be substrate specific. With some substrates PDI displayed no activity or even a negative, anti-chaperone activity [32]. The peptide-binding domain of PDI is shown to be responsible for the chaperone activity. Experiments in *E. coli* have shown that the deletion of the C-terminal c-domain of PDI abolishes the peptide binding properties and the chaperone activity of PDI, whereas most of its catalytic activities for isomerization are retained [33].

6. Glycosylation

A major biosynthetic function of the ER is the N-glycosylation of proteins. N-linked oligosaccharides play multiple roles in the folding process of glycoproteins. They are needed to stabilize folded domains and make the protein more soluble, which prevents aggregation of folding intermediates. Furthermore, they are responsible for the interaction of the newly synthesized glycoprotein with the resident ER lectin chaperones calnexin and

calreticulin [34]. Besides the role of the N-linked glycans in folding, they have many biological functions in the cell. They serve in intracellular targeting, they modulate clearance rates of proteins from blood, and they are involved in numerous recognition-related processes on the cell surface [35].

The addition of oligosaccharides is a co-translational event. As soon as the asparagine residue, in the consensus sequence N-X-S/T (where X is any amino acid except proline), emerges into the ER-lumen, the oligosaccharide $GlcNAc_2Man_9Glc_3$ is added *en bloc* to the translocated protein by a membrane bound oligosaccharyl transferase. Immediately after the attachment of the oligosaccharide to the nascent chain, the three terminal glucose residues are trimmed, followed by the removal of on or two mannose residues. When the protein is properly folded it is transported to the Golgi complex.

When N-linked glycosylation is inhibited, the folding of several glycoproteins fails. Misfolded proteins aggregate and associate non-covalently with BiP, an abundant molecular chaperone. Eventually they will be degraded [36]. Most glycoproteins need their glycans for proper folding but this dependence is variable. Whereas some glycoproteins cannot fold at all, some proteins show partial misfolding of a fraction of the molecules with the rest folding correctly and leaving the ER [37]. Another set of glycoproteins does not need their N-linked glycans at all for proper folding [38]. Not all N-linked glycosylation sites are necessary: often deletion of one site does not interfere with correct folding, but multiple deletions of N-linked glycosylation sites often do so [39].

The extent and timing of trimming of the oligosaccharides in the ER may interfere with the folding process. Inhibitors of glucosidase I and II often delay or even prevent the folding and transport of newly synthesized glycoproteins in the ER [40]. The trimming of the glucose residues is tightly linked to ER quality control: the monoglucosylated core oligosaccharide is a substrate for the molecular chaperones calnexin and calreticulin [41]. Mannose trimming, on the other hand, is associated with degradation, since glycoproteins carrying the $GlcNAc_2Man_8$ sugar are recognized by a lectin that targets them for degradation [42, 43]. Additional lectins, with affinity for other transiently existing carbohydrates in the ER, may emerge in the near future.

7. Calnexin and calreticulin

Virtually all glycoproteins associate during their biosynthesis with the lectin chaperones calnexin and calreticulin in the ER [44]. Calnexin is a type I membrane protein that was first identified as a major calcium binding protein of the mammalian ER [45]. Calreticulin is a soluble ER resident lectin-like chaperone and is closely related to calnexin. They bind N-linked glycosylated proteins, and then exclusively the monoglucosylated forms [36]. This association is independent of the tertiary structure of the protein moieties [44]. Both calnexin and calreticulin can promote folding, oligomerization and suppress degradation as demonstrated for the influenza virus hemagglutinin glycoprotein [46]. Calnexin and calreticulin are thought to exclusively bind via the glycan portion on proteins, but Williams and colleagues suggest that calnexin associates with non-glycosylated proteins as well [47]. Using in vitro assays, they showed that calnexin can inhibit the aggregation of non-glycosylated proteins in an ATP-dependent fashion [47].

Together with the enzymes glucosidase II and UDP-glucose: glycoprotein glucosyltransferase (GT), calnexin and calreticulin regulate the retention of misfolded and not-yet-folded glycoproteins in the ER [36]. When properly folded, the glycoprotein can leave the ER and follow the route to its final destination. When the glycoprotein is misfolded or not folded yet, however, it is detected as such by GT, which adds a glucose residue back onto the core glycan. The glycoprotein associates again with calnexin and calreticulin, which completes one cycle of association and release. In this model, GT is the quality sensor for proteins, and the lectin chaperones are responsible for retention in the ER. GT recognizes the oligosaccharide, specifically the N-acetylglucosamine, and hydrophobic stretches on the misfolded moiety of the glycoprotein [48]. This cycle represents an important quality control mechanism in the ER which allows only properly folded glycoproteins to exit the organelle [16]. Molinari and Helenius suggested that timing of the association of calnexin and calreticulin with substrate glycoproteins is determined by the position of the N-linked glycan within the polypeptide chain [49]. When proteins contain N-linked glycans within their first ~50 residues they interact with calnexin and calreticulin prior to their subsequent binding to BiP or other chaperones [50]. Examples are the SFV protein E, HIV-1 envelope glycoprotein gp160 and immunoglobulin heavy chain. Although this hypothesis may be true for many glycoproteins, there are exceptions, e.g., thyroglobulin [51]. Calnexin and calreticulin form a complex together with the oxidoreductase ERp57 [52, 53].

8. N-linked glycosylation of gp160

Heavily glycosylated proteins require their glycans for proper folding. Gp160 is no exception: when certain glycosylation sites are deleted, the protein will not leave the ER [54]. On the other hand, some N-linked glycosylation sites are dispensable for gp160 folding, as determined by its capability to bind CD4 and to induce syncytia [55]. Non-glycosylated forms of gp120 generated either by deletion of the signal sequence of HIV-1 gp120 or by synthesis in the presence of tunicamycin [40] failed to

bind to CD4 [56]. Our results showed complete disulfide-linked aggregation and misfolding of gp160. This importance of glycans for gp160 folding and structure is no surprise, considering their contribution to the total mass of the protein (almost 50% in gp120 (*figure 1B*)).

Maximum association of calnexin occurred around 10 min. after the start of synthesis, whereas calreticulin association peaked at 5 min after synthesis. Calnexin and calreticulin bound exclusively to gp160 folding intermediates: as folding proceeded, gp160 was released (our unpublished results). Most of the gp160 molecules bound to calnexin were also bound to calreticulin in a transient ternary complex, whereas some gp160-calnexin complexes existed without calreticulin [57].

9. BiP

BiP is an abundant lumenal ER chaperone of 78 kDa, which belongs to the hsp70 family. Besides sealing the translocon on the lumenal side of the ER, BiP functions as a molecular chaperone. BiP was first identified in association with Immunoglobulin heavy chains [58]. The peptide-binding sites of BiP are responsible for the recognition of exposed sections on unfolded proteins [59]. BiP in addition has an ATP binding domain; ATP hydrolysis is coupled to peptide binding and release. The dissociation of several other chaperones from the target protein requires the hydrolysis of ATP as well. BiP binds to virtually every misfolded protein tested [20]. In addition BiP was reported to be involved in the targeting of misfolded proteins for degradation [60].

Gp160 was found to interact with BiP, perhaps already during association with the lectin chaperones. BiP dissociation occurs at around the same time as calnexin and calreticulin dissociation, before the formation of the native conformation of gp160 [10]. Potential BiP binding sites are located within the conserved regions of gp160 and gp120; no binding sites for BiP were located in the hypervariable regions of gp120 [61]. This would fit with our observations that the conserved domains fold prior to the variable domains.

10. Conclusion

The folding process of HIV-1 gp160 in the ER differs from that observed for other proteins. The most notable difference is the post-translational removal of the signal peptide, and its relation with conformational maturation of the protein. Another observed difference is the relative independence of folding rate on the cellular environment. Apparently the folding is determined more by the protein itself than by the cell, as long as the conditions do support proper folding. The gp160 molecule is an extremely slowly folding protein, even though it is of viral origin. Perhaps its large number of glycans is the cause of this low folding rate. It undergoes the folding process under guidance of at least a few highly abundant chaperones and folding enzymes. This set of assistants is likely to be much larger than we know. Continued searches for ER folding factors one day may yield factors or a combination thereof, that are so specific that they will be useful targets for antiviral therapy.

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