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Glycoprotein Tertiary and Quaternary Structures Are Monitored by the Same Quality Control Mechanism*

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Folding of glycoproteins entering the secretory pathway is strictly surveyed in the endoplasmic reticulum by a quality control system. Folding intermediates and proteins irreparably misfolded are marked *via* glucosylation by the UDPglucose:glycoprotein glucosyltransferase, an enzyme that acts as a folding sensor by exclusively labeling glycoproteins not displaying their native structures. Here we show that this sensing mechanism also applies to the oligomerization of protein complexes, as the glucosyltransferase appeared to be able to glucosylate folded complex subunits lacking the full complement of oligomer components.

Newly synthesized proteins must complete folding before they move through the secretory pathway. A quality control mechanism retains misfolded and unassembled species in the endoplasmic reticulum (ER)[1] (1, 2). This system is composed of four main elements, the ER resident lectins calnexin and calreticulin and the enzymes glucosidase II and UDPglucose:glycoprotein glucosyltransferase (GT). About 80% of proteins entering the secretory pathway are *N*-glycosylated cotranslationally in the consensus sequence Asn-*X*-(Ser/Thr). The two outermost glucoses are removed from the transferred glycan ($Glc_3Man_9GlcNAc_2$) by the sequential action of glucosidase I and glucosidase II. The monoglucosylated species thus generated are then recognized by calnexin and/or calreticulin, which are lectins specific for $Glc_1Man_{5–9}GlcNAc_2$ glycans (3). The glycoprotein-lectin association not only retains in the ER the species that are not properly folded but also enhances folding efficiency by preventing the aggregation of intermediates and allowing the action of other folding-enhancing proteins such as ERp57, a protein disulfide isomerase associated with calnexin and calreticulin (4). The glycoprotein-lectin interaction is eventually terminated upon glucosidase II cleavage of the remaining glucose. At this stage, glycoproteins that are properly folded proceed to their final destination. Folding intermediates or irreparably misfolded glycoproteins, however, are reglucosylated by GT, an enzyme that recreates the monoglucosylated structure, and are thus retained by the lectins in the ER. GT operates as a folding sensor, because it only glucosylates glycoproteins not displaying their native structures (5). Protein determinants triggering GT-mediated glucosylation have been identified as patches of hydrophobic amino acids that are mainly solvent-exposed in molten globule-like conformations (6).

It has been observed *in vivo* that only fully assembled oligomeric proteins leave the ER. The above mentioned glycoprotein folding quality control mechanism has been implicated in the retention of individual subunits or partially assembled oligomers, because those species have been isolated as being associated to calnexin/calreticulin from cells unable to form the complete oligomers (7, 8). For instance, the lectin-glycoprotein association of T cell receptor or human class I major histocompatibility complex components has been observed to last until the complete assembly of the multimeric complexes (9, 10). The work reported here shows that GT is sensitive enough to sense hydrophobic patches exposed in interfaces of not yet assembled but otherwise native-like folded subunits and that the lectin subunit or lectin incomplete complex association is not necessarily determined by GT-mediated glucosylation of incompletely folded individual subunits.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were purchased from Sigma. The Superdex 200 column was acquired from Amersham Biosciences. Rat liver GT was purified as described (11). Soybean agglutinin (SBA) was purified from untoasted soybean meal by affinity chromatography on acid-treated Sepharose 6B (12).

Spectroscopic Characterization of SBA—CD spectra were measured with a Jasco J810 spectropolarimeter. SBA concentration was 1.8 μM in far-UV and 18 μM in near-UV CD. Intrinsic fluorescence and 8-anilinonaphthalene-1-sulfonic acid (ANS) binding were measured with an Aminco Bowman series 2 spectrofluorometer with 18 μM SBA. We used 50 μM ANS with the excitation wavelength set at 370 nm, and spectra were recorded from 400 to 600 nm. All spectra were recorded at 25 °C in 40 mM HEPES and 20 mM KCl, pH 7.4 (buffer A) with the addition of 0.2 mM EDTA

Protein Denaturation—To eliminate aggregates, SBA was further purified before use by size exclusion chromatography in a Superdex 200 eluted with buffer A at a flux rate of 0.5 ml/min. SBA (18 μM) was dissolved in the same buffer at the indicated urea concentrations with the addition of 0.2 mM EDTA. SBA concentration was determined from the absorbance at 280 nm using $A^{1\%,\ 1cm} = 12.8$ and expressed in terms of monomer (M_r = 30,000). Samples were incubated for 18 h at 37 °C to ensure the achievement of equilibrium. Fluorescence spectra were measured with the excitation wavelength set at 280 nm, and spectra were recorded from 300 to 430 nm. Excitation and emission band pass values were both 5 nm. The process was followed by assessing the fluorescence spectrum center of mass.

Size Exclusion Chromatography—To verify the appearance of the monomer, size exclusion chromatography of SBA was performed at 25 °C in a Superdex 200 column with buffer A with the addition of 0.2 mM EDTA and containing the same urea concentration of the sample. The flow rate employed was 0.5 ml/min, and the eluted material was monitored by the absorbance at 280 nm.

Activity Assays—GT assays were carried out at 37 °C in 120 μl of buffer A with the addition of 5 mM $CaCl_2$ and 15 μM UDP-glucose

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[1] The abbreviations used are: ER, endoplasmic reticulum; ANS, 8-anilinonaphthalene-1-sulfonic acid; CD, circular dichroism; GT, UDPglucose:glycoprotein glucosyltransferase; SBA, soybean agglutinin.

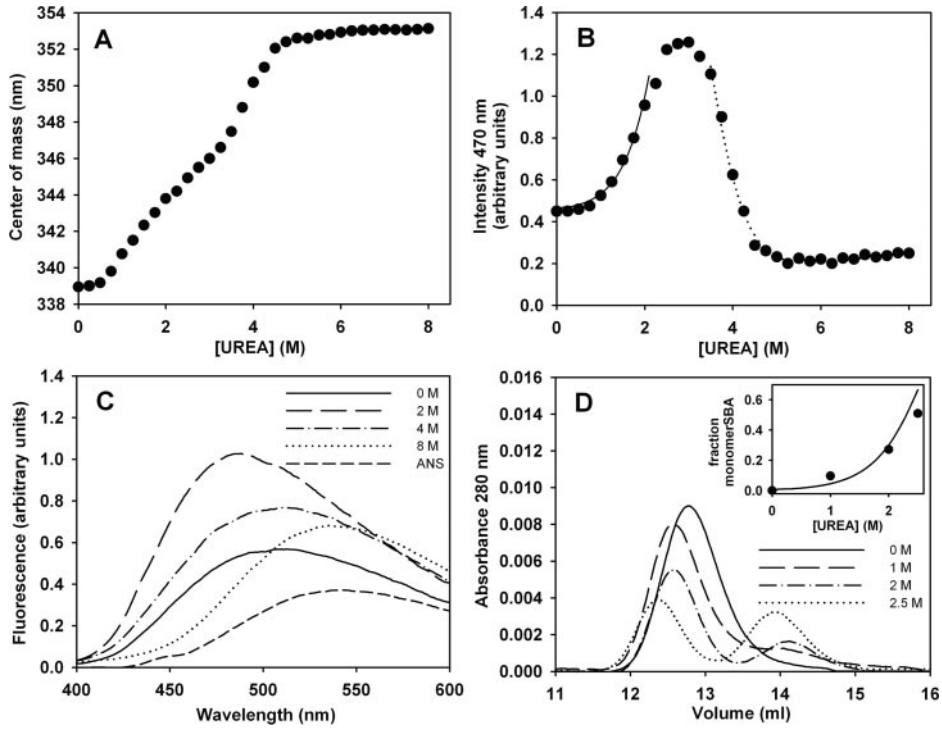

FIG. 1. **Urea-induced denaturation of SBA.** *A*, intrinsic fluorescence center of mass. *B*, ANS fluorescence intensity at 470 nm. The *lines* denote the predicted ANS fluorescence values for the dissociation of the tetramer (*solid line*) and the denaturation of the monomer (*dotted line*). Urea concentrations at midpoints are 2.4 and 3.5 M, respectively. The model assumes a linear dependence of the process's free energy with denaturant concentration (13). *C*, ANS fluorescence spectrum at representative urea concentrations. *D*, SBA disassembly followed by size exclusion chromatography. *Inset*, fraction of monomeric SBA derived from the peaks corresponding to tetramer and folded monomer SBA. The *line* represents the fraction of folded monomer SBA predicted from the model used in Fig. 2*B*. Urea concentrations are indicated *below the graph*.

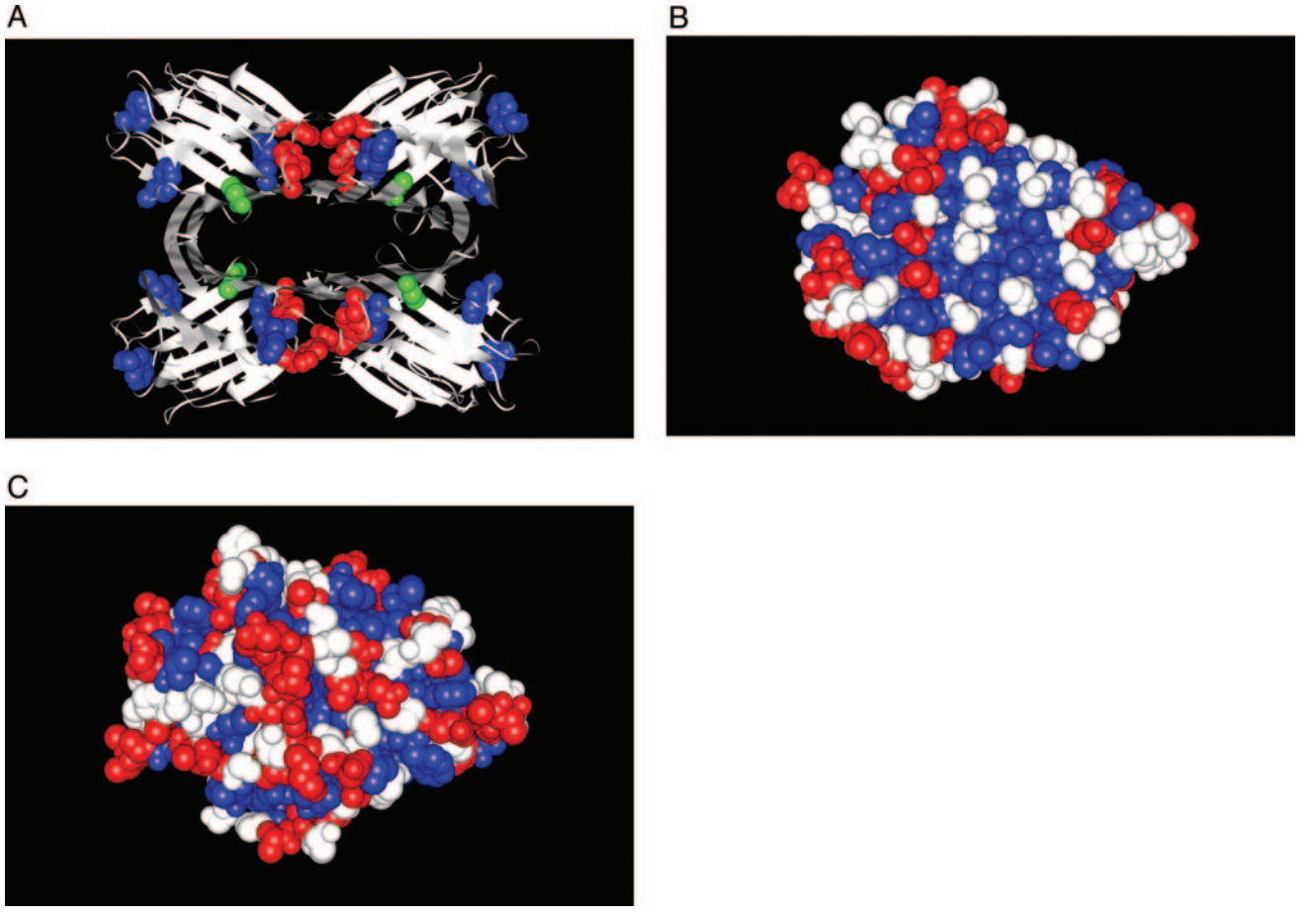

FIG. 2. **SBA structure.** *A*, tetrameric SBA. *Blue*, tryptophan residues; *red*, tryptophan residues in the interface between subunits; *green*, Asn-75 (*N*-glycosylation site). *B*, the monomer surface facing the tetramer, with hydrophobic residues (Ala, Ile, Phe, Leu, Met, Trp, and Tyr) colored *blue*, strong hydrophilic residues (Glu, Asp, Lys, Asn, Gln, and Arg) colored *red*, and the remaining residues colored *white*. *C*, the monomer surface facing the solvent is colored as described for *panel B*.

(300,000 cpm UDP-[^{14}C]glucose). Reactions were started by dilution of SBA to a final concentration of 0.9 μM in the reaction buffer containing 4 μg of rat liver GT. To allow comparison between samples, urea was added to a 140 mM final concentration in all samples. Reactions were stopped after 4 min by the addition 30 μl of cracking buffer at 100 °C containing 5% SDS. Samples were immediately treated for 10 min in a

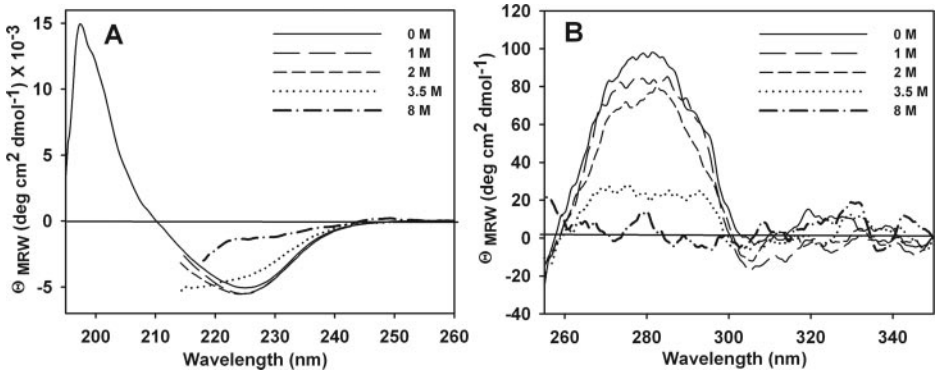

FIG. 3. **Circular dichroism spectroscopy.** *A*, far-UV CD. *B*, near-UV. Urea concentrations are indicated in the *insets*. θ_{MRW}, molar ellipticity.

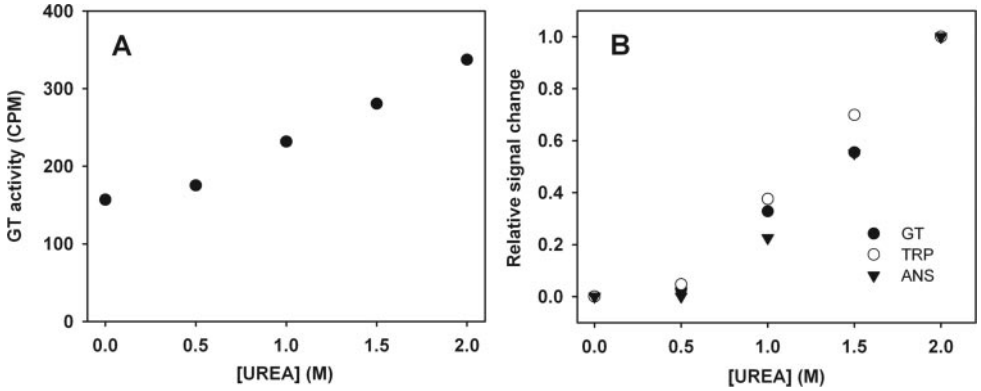

FIG. 4. **Glucosylation of subunits.** *A*, dependence of GT activity on urea concentration. *B*, normalized relative changes of GT activity with tryptophan fluorescence center of mass and ANS fluorescence intensity at 470 nm.

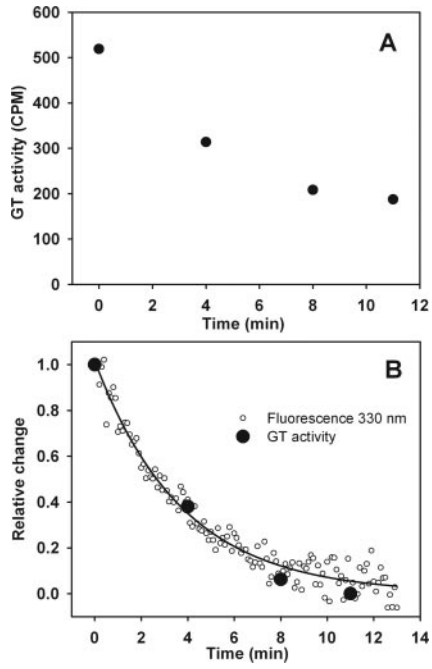

FIG. 5. **Reassociation of SBA monomers.** *A*, GT activity. *B*, normalized relative change of GT activity and tryptophan fluorescence intensity at 330 nm. *Line* denotes single exponential fitting of the fluorescence data.

heat block at 90 °C and concentrated to ~40 μl in a SpeedVac. Samples were then subjected to electrophoresis in 12.5% SDS-polyacrylamide gels. A low energy phosphor screen (Amersham Biosciences) was exposed to the dried gel for 2 days and revealed in a Storm 820 PhosphorImager. Under these conditions, a linear relationship between density and radioactivity was obtained. For renaturation experiments, 25 μl of SBA (18 μM) in 2.5 M urea was diluted in 775 μl of 40 mM HEPES pH 7.4, 0.2 mM $CaCl_2$, and 0.2 mM $MnCl_2$ at 20 °C. Aliquots of 75 μl were withdrawn at the indicated times and diluted to assay GT activity in a 110-μl final volume as described above. Reactions lasted for 3 min at 20 °C. Renaturation was followed in parallel at the same temperature by following tryptophan fluorescence. In this experiment the excitation and emission wavelengths were 280 and 330 nm, with a band pass of 0.5 and 16 nm, respectively.

RESULTS AND DISCUSSION

Generation of Monomeric SBA—A suitable model to study the dependence of GT activity on the substrate quaternary structure requires a high mannose-type multimeric glycoprotein, the individual subunits of which preserve their tertiary structures upon oligomer disassembly. SBA, an oligomeric protein formed by four identical glycoprotein subunits, fulfills this condition. Chemical denaturation with urea followed by the fluorescence center of mass showed the presence of an intermediate at ~2 M concentration (Fig. 1*A*) (13). This result was further confirmed by following ANS fluorescence (Fig. 1*B*). This compound is a probe sensitive to the presence of exposed hydrophobic patches. The ANS fluorescence spectrum had an increased intensity and was shifted to shorter wavelengths at 2 M urea, indicating exposition of a wider hydrophobic surface as compared with the initial state (Fig. 1*C*). Analysis by size exclusion chromatography confirmed the appearance of a smaller protein with a molecular weight corresponding to that of the monomer (Fig. 1*D*). SBA has six tryptophan residues, two of them (Trp-8 and Trp-203) located in the interface between subunits (Fig. 2*A*). The red shift of the protein fluorescence reflects exposition of those particular residues following the tetramer disassembly. Upon this process, SBA exposes an additional 1325 $Å^2$ of accessible surface area per monomer, of which 873 $Å^2$ correspond to a hydrophobic surface (Fig. 2, *B* and *C*) (14). Exposition of this surface during the first transition (tetramer to monomer) explains the increased ANS binding. The far-UV CD spectrum remained fairly constant along the first transition (Fig. 3*A*), thus indicating that the individual subunits preserved the antiparallel β-sheet conformation they

displayed in the tetramer. On the other hand, only a minor change was observed in the near-UV CD (Fig. 3*B*). At this wavelength, CD analysis mainly reflects variations in the asymmetry of the environments of the tryptophan residues located on the interfaces between subunits. Drastic changes in both far- and near-UV CD spectra were observed at urea concentrations higher than 3.5 M, thus reflecting the unfolding of the monomer, the second transition (Fig. 3, *A* and *B*). In summary, SBA dissociates at low urea concentrations into monomers that expose the hydrophobic interfaces hidden between subunits but that otherwise preserve the secondary and tertiary structures displayed in the tetramers.

GT Senses the Oligomeric State of SBA—To study whether GT is able to sense the oligomerization status of glycoproteins, we assayed the enzymatic activity along the first transition. Urea-treated SBA samples were diluted, and GT activity was determined. Incubation times were much shorter than those required for tetramer assembly completion (see below). The catalytic activity was constant up to 0.5 M urea and then increased linearly with urea concentration (Fig. 4*A*). This behavior paralleled changes in tryptophan and ANS fluorescence (Fig. 4*B*). Moreover, a linear correlation of GT activity with tryptophan and ANS fluorescence was observed (correlation coefficients of 0.977 and 0.985 respectively; data not shown). These results show that GT activity followed the disassembly of the tetramer. As mentioned above, in monomeric glycoproteins GT recognizes exposed hydrophobic residues. This process is more efficient for advanced folding intermediates such as molten globules, where the exposed hydrophobic residues form patches (6). Because the secondary and tertiary structures of the monomer were preserved during SBA disassembly, the observed increase in GT activity was a consequence of the exposition of the hydrophobic interface between subunits.

To study how the assembly process of an oligomer in the ER affected recognition by GT, we measured the activity after dilution of monomeric SBA in a reconstituting buffer. GT activity diminished exponentially with time (Fig. 5*A*). This behavior mirrored the changes in tryptophan fluorescence, indicating that the loss of GT activity was coupled to SBA assembly (Fig. 5*B*). Again, changes in enzymatic activity appeared to be a consequence of the reduction in the exposed hydrophobic surface upon the transition of monomers to tetramer.

It may be argued that GT glucosylation could be triggered not by exposure of the hydrophobic interface between subunits but by slight modifications of the subunit tertiary structures, not detected by CD spectra, that are caused by the presence of urea in the dissociation buffer. This possibility, however, seems highly unlikely because the bottleneck in tetramer formation is not the refolding of subunits but oligomer assembly. Thus, it has been determined that the transition of a fully denatured monomer into a folded monomer takes less than 10 s, whereas the oligomerization process is completed only after ~15 min (15). It would be expected, therefore, that upon diluting SBA in reconstitution buffer the minimal imperfections in the subunit tertiary structure would disappear within a time period not significantly influencing GT activity.

The synthesis of oligomeric proteins poses additional problems to the cell compared with their monomeric partners. The relative concentration of each subunit may be regulated according to the stoichiometry of the protein complex. However, these regulatory mechanisms would be ineffective in a deficient assembly process. Moreover, the presence of a protein subunit free from its parent complex can have serious consequences, because the exposure of the surface normally hidden in the oligomer may trigger an aggregation process (16). For instance, patients on long-term hemodialysis can develop amyloidosis generated by β2-microglobulin free from a class I major histocompatibility complex heavy chain (17). The glycoprotein quality control system could have evolved, among other reasons, to avoid these situations. If the definitive tertiary structure of the subunits is acquired upon oligomer assembly, individual subunits are expected to be glucosylated by GT until they form the final complex, as GT appeared to be able to glucosylate glycoprotein monomers displaying structures minimally differing from native ones (18). The work presented here suggests that, on the other hand, if the tertiary structure is attained before assembly, GT will recognize the subunit interface of the partially assembled species. As a result of a remarkable cellular economy when establishing quality control devices, in both scenarios an identical mechanism will retain intermediate species in the ER until folding and/or assembly processes are completed.

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