

Quaternary and Domain Structure of Glycoprotein Processing Glucosidase II[†]E. Sergio Trombetta,^{*,‡} Karen G. Fleming,[§] and Ari Helenius^{||}

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ABSTRACT: Glucose trimming from newly synthesized glycoproteins regulates their interaction with the calnexin/calreticulin chaperone system. We have recently proposed that glucosidase II consisted of two different subunits, α and β . The α subunit is the catalytic component, and deletion of its homologue in yeast obliterates glucosidase II activity. Deletion of the homologue of the noncatalytic β subunit in *Schizosaccharomyces pombe* drastically reduces glucosidase II activity, but the role of the β subunit in glucosidase II activity has not been established. Furthermore, a direct interaction between α and β subunits has not been demonstrated. Using chemical cross-linking and hydrodynamic analysis by analytical ultracentrifugation, we found that the two subunits form a defined complex, composed of one catalytic subunit and one accessory subunit ($\alpha_1\beta_1$) with a molecular mass of 161 kDa. The complex had an *s* value of 6.3 S, indicative of a highly nonglobular shape. The asymmetric shape of the $\alpha_1\beta_1$ complex was confirmed by its high susceptibility to proteases. The β subunit could be proteolytically removed from the $\alpha_1\beta_1$ complex without affecting catalysis, demonstrating that it is not required for glucosidase II activity in vitro. Furthermore, we isolated a monomeric C-terminal fragment of the α subunit, which retained full glucosidase activity. We conclude that the catalytic core of glucosidase II resides in a globular domain of the α subunit, which can function independently of the β subunit, while the complete α and β subunits assemble in a defined heterodimeric complex with a highly extended conformation, which may favor interaction with other proteins in the endoplasmic reticulum (ER). Through its C-terminal HDEL signal, the β subunit may retain the complete $\alpha_1\beta_1$ complex in the ER.

Protein N-glycosylation is initiated in the lumen of the endoplasmic reticulum (ER) by the transfer of a preassembled oligosaccharide (Glc₃Man₉GlcNAc₂) from a lipid precursor to Asn residues in nascent polypeptide chains (1). Immediately after being added to growing polypeptides, oligosaccharides are trimmed by glucosidase I, a type II transmembrane protein of the ER (2). The resulting Glc₂-Man₉GlcNAc₂ structures are further trimmed by glucosidase II. Removal of the outermost α -1,3-linked Glc residue generates Glc₁Man₉GlcNAc₂, which allows newly synthesized glycoproteins to interact with calnexin and calreticulin, two lectins specific for monoglucosylated oligosaccharides. These interactions and the consequent exposure to the oxidoreductase ERp57 are part of a chaperone mechanism that assists the correct folding and oligomerization of glycoproteins in the ER (3).

The monoglucosylated glycans recognized by calnexin and calreticulin can also be generated by a luminal ER enzyme that reglucosylates fully deglucosylated structures (Man₅₋₉-GlcNAc₂) to regenerate the corresponding Glc₁Man₅₋₉GlcNAc₂

(4). The glucosyltransferase selectively reglucosylates glycoproteins that have not attained a fully folded conformation. In this way, a terminal Glc residue is cyclically removed and re-added to incompletely folded glycoproteins through the opposing action of the glucosyltransferase and glucosidase II (3). These two enzymes are ubiquitously expressed soluble residents of the ER. The monoglucosylated structures (Glc₁Man₅₋₉GlcNAc₂) are also deglucosylated by glucosidase II to generate glycoproteins bearing Man₅₋₉GlcNAc₂ structures.

We have recently proposed that rat liver glucosidase II is composed of two different polypeptides, and we called them subunits α and β . On the basis of its homology to other glycosidases, the α subunit was identified as the catalytic component, showing maximal similarity to the family 31 glycoside hydrolases (5, 6). Deletion of the homologous gene in yeast results in complete lack of glucosidase II activity (7–12). The catalytic subunit had no apparent transmembrane domains, in agreement with its behavior as a soluble protein. However, it had no KDEL-related (HDEL) sequences at its C-term, showing no apparent ER retention signal. This was puzzling since glucosidase II has to act on its substrates in the ER, where it has been localized by immunoelectron microscopy (13).

The cDNA for the β subunit encodes a polypeptide with no homology to other known proteins, and no clear function could be predicted from its sequence (7). Because it contains a KDEL sequence at the C terminus, we predicted that it

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would associate with the α subunit and mediate retention of an α/β oligomer in the ER. Indirect evidence for the existence of such a complex came from genetic experiments in *S. pombe*, where deletion of the gene-coding β subunit drastically reduced glucosidase II activity in vivo (14). In mammalian cells, the requirement for the β subunit has been less clear since increased glucosidase II activity was obtained by overexpression of the α subunit alone (9) or by coexpression with the β subunit (9, 11, 12), which was in turn found to coprecipitate some of the endogenous glucosidase II activity (15).

While the in vivo experiments indicate that the β subunit influences glucosidase II activity, they do not demonstrate a direct interaction between the α and the β subunits, since the effects observed could be explained by indirect mechanisms. Also, while we found that both subunits copurified (7), other groups described glucosidase II as a single polypeptide consisting of the catalytic α subunit alone (9, 16–18). Because no direct experimental evidence has been provided so far to demonstrate the existence of an α/β complex or to characterize its composition, we analyzed the behavior of glucosidase II in solution. We found that one α and one β subunit assemble into a well-defined stable complex with a 1:1 stoichiometry. The $\alpha_1\beta_1$ complex was highly nonglobular and markedly sensitive to proteases, allowing us to proteolytically remove the β subunit to obtain samples in which the α subunit remained fully active, demonstrating that the β subunit is not required for glucosidase activity in vitro. These results, together with previous reports on the effect of expression of the β subunit, suggest that assembly of an $\alpha_1\beta_1$ complex in vivo is necessary for glucosidase II activity.

MATERIALS AND METHODS

Materials. Chemicals were from Sigma (St. Louis, MO) unless otherwise indicated. The chromatographic media were from Pharmacia (Piscataway, NJ). Rat liver glucosidase II was purified as previously described (7). Glucosidase II activity was measured in a final volume of 50–100 μ L, containing 1 mM *p*-nitrophenyl- α -D-glucopyranoside, 150 mM NaCl, and 10 mM HEPES (pH 7.5) or 10 mM Tris-HCl (pH 7.5). Reactions were started by adding glucosidase II preparations, incubated at 37 °C for the indicated period of time, and stopped by the addition of one reaction volume (50–100 μ L) of 2 M Tris base. Glucose release was estimated by the production of *p*-nitrophenol, measured by absorbance at 405 nm.

Cross-Linking Experiments. Rat liver glucosidase II (2 μ g) was incubated in a final volume of 40 μ L in the presence of 5% dimethyl sulfoxide (DMSO) in TBS (20 mM Tris-HCl and 150 mM NaCl, pH 7.4) or in the presence of 0.2 mg/mL DSP (dithiobis[succinimidyl propionate]; Pierce, Rockford, IL) or 0.2 mg/mL DSG (disuccinimidyl glutarate; Pierce, Rockford, IL) in 5% DMSO. After 10 min at 25 °C, 10- μ L aliquots from each incubation were mixed with sample buffer with or without 20 mM dithiothreitol (DTT), heated for 10 min at 95 °C, and run on 6% SDS-PAGE stained with Coomassie blue.

Sedimentation Equilibrium. A Beckman XL-I analytical ultracentrifuge was used to obtain the equilibrium distribution of the glucosidase II complex at three initial concentrations

and at three speeds in phosphate-buffered saline. The protein concentration was detected using the absorbance optics at 280 nm. Three initial concentrations were evaluated ranging from 0.1 to 0.8 mg/mL at 20 °C. Rotor speeds included 10 800, 13 300, and 16 300 rpm. Sample analysis was carried out using standard six-sector cells equipped with quartz windows and sample volumes of 110 μ L. Sedimentation equilibrium was established by subtraction of subsequent scans until the difference was equal to the noise of the system. The MacIntosh version of NONLIN algorithm (19) was used to analyze sedimentation equilibrium data in order to obtain estimates for the molecular weight and association states. A value of 0.7250 mL g⁻¹ for the protein partial specific volume of the 1:1 complex was calculated from the amino acid composition using SEDNTERP. A solvent density of 1.006 59 g mL⁻¹ was also calculated using SEDNTERP.

Sedimentation Velocity. Sedimentation velocity data were collected at 20 °C at 45 000 rpm using the absorbance optics to visualize the protein. The sample and buffer volumes were 450 μ L. Initial concentrations ranged from 0.1 to 0.8 mg/mL. Scans were collected at the maximum allowed rate of data collection. Data were analyzed according to the time derivative method of Stafford (20–22) using the DCDT software program to yield a sedimentation coefficient distribution, $g(s^*)$, from which a weight average sedimentation coefficient at a particular concentration and temperature can be calculated by integration over the distribution function with respect to s^* as described previously by Stafford (20–22). The $s_{20,w}$ was calculated from the experimentally observed sedimentation coefficient using SEDNTERP. The frictional coefficient, $f_{20,w}$, was calculated from the $s_{20,w}$ using the Svedberg equation. SEDNTERP was used to estimate a value for hydration ($\delta = 0.4344$) from the amino acid composition. The programs SEDNTERP, NONLIN, and DCDT are available as freeware from the RASMB web site at <http://www.bbri.org/rasmb/rasmb.html>.

Limited Proteolysis. For the analytical scale proteolysis experiments shown in Figure 4, rat liver glucosidase II (4 μ g) was incubated at 0 or 25 °C with the indicated proteases for 5–60 min at protease/glucosidase ratios (1:50–1:20 000) in a final volume of 40 μ L of TBS buffer. Incubations were stopped by addition of Laemmli sample buffer (with or without 10 mM DTT) and heated at 100 °C for 5 min. *N*-Ethylmaleimide was added to a final concentration of 20 mM to all samples, and they were then analyzed by 12.5 or 15% SDS-PAGE and protein detected by Coomassie blue staining.

For trypsin digestion, rat liver glucosidase II (10 μ g) was incubated with trypsin (2–20 μ g/mL) at 25 °C in a final volume of 50 μ L of TBS buffer. At the indicated time points, 10- μ L aliquots were removed and mixed with 1 μ L of TBS containing soy bean trypsin inhibitor (1 μ g) on ice. From each sample, 2 μ L was used to measure glucosidase activity in duplicates, and 5 μ L was run on SDS-PAGE stained with Coomassie blue. For larger scale trypsin digestion, rat liver glucosidase II (100 μ g) was incubated with trypsin (10 μ g/mL) in a total volume of 500 μ L of TBS buffer. After 1 h at 25 °C, 50 μ g of soy bean trypsin inhibitor was added, and the mixture was run on Superose12, equilibrated, and developed in TBS buffer at 0.5 mL/min in a cold room. Fractions of 400 μ L were collected and assayed for glucosi-

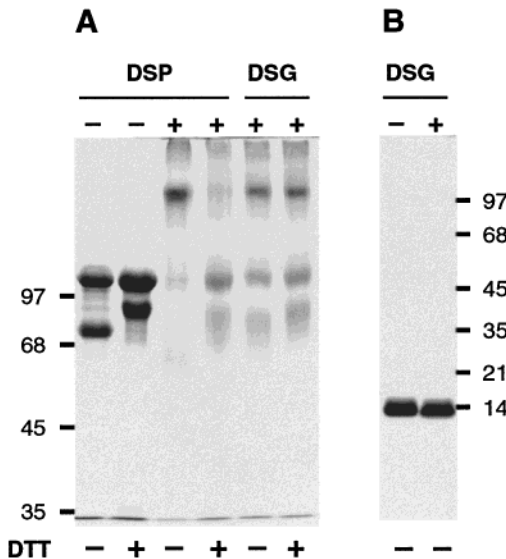


FIGURE 1: α and β subunits of rat liver glucosidase II can be readily cross-linked. Rat liver glucosidase II was subjected to chemical cross-linking with the indicated compounds (lanes 1–6 from left to right). The top band in lane 1 at around 110 kDa corresponds to the α -subunit, while the lower band corresponds to the β -subunit, both in reducing and nonreducing conditions. After 10 min at 25 °C, the reaction mixtures were boiled in sample buffer with or without DTT as indicated and separated in 6% SDS–PAGE stained with Coomassie blue. As a control for nonspecific cross-linking, hen egg lysozyme was incubated under the same conditions, boiled in nonreducing sample buffer, and analyzed in 15% SDS–PAGE (lanes 7 and 8).

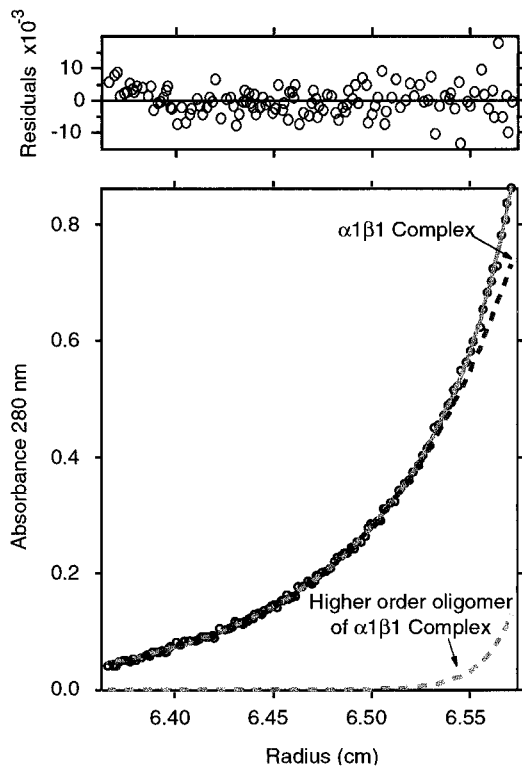


FIGURE 2: Sedimentation equilibrium analysis of rat liver glucosidase II. The absorbance vs radius profile for glucosidase II complex (0.2 mg/mL) at 13 300 rpm is shown in the lower panel. The open circles are data points, and the solid line is the model fit described by eq 1. The two dotted lines whose sum gives rise to the fit represent the distributions of monomeric $\alpha_1\beta_1$ complex and a small amount of higher order species. The residuals of the fit to eq 1 are shown in the upper panel.

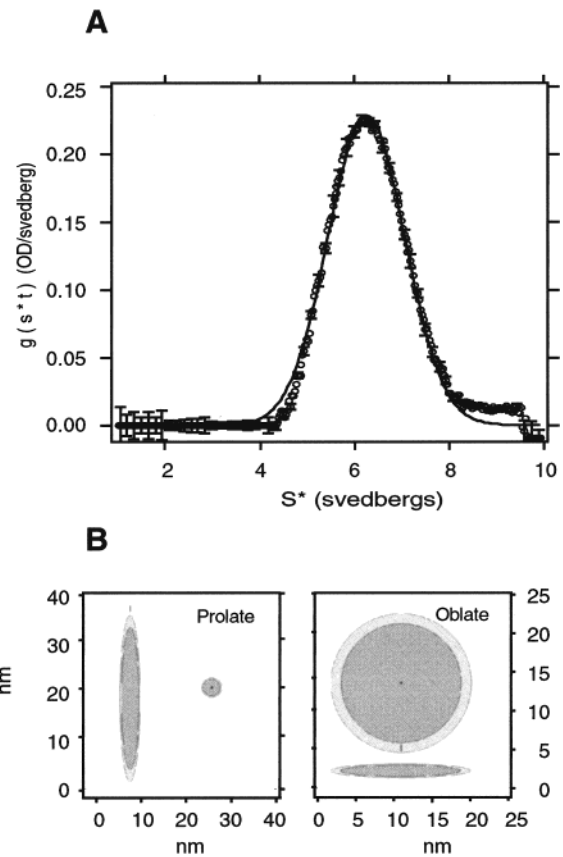


FIGURE 3: Sedimentation velocity analysis of rat liver glucosidase II. (A) Apparent sedimentation coefficient distribution function, $g(s^*)$ vs s^* , for glucosidase II complex (0.2 mg/mL) at 45 000 rpm. The error bars represent the standard error of the mean of the 10 data sets used in this analysis. For clarity, only every fifth error bar is shown. The solid line demonstrates a good fit to a single Gaussian function. (B) Scaled models for the axial ratios of the elongated shapes calculated for the $\alpha_1\beta_1$ complex generated using the standard calculations implemented in SEDNTERP. Both top and side views are shown for the ellipsoids of revolution models with hydration expansions of 17%. Prolate model: $a/b = 8.2$, $2a = 34.2$, and $2b = 4.1$ nm. Oblate model: $a/b = 9.7$, $2a = 17.9$, and $2b = 1.8$ nm.

dase activity and analyzed by SDS–PAGE stained with Coomassie blue. For N-terminal sequencing, gels were transferred to PVDF membranes (Millipore), and the bands containing between 50 and 200 ng (based on comparison to bovine albumin standards) were excised and subjected to automated Edman degradation on a Procise CLC automated amino acid sequencer (Applied Biosystems, Foster City, CA).

RESULTS

Quaternary Structure of Glucosidase II. To investigate whether the α and β subunits of rat liver glucosidase II were directly interacting with each other, we submitted the intact enzyme to chemical cross-linking. As shown in Figure 1A, both subunits could be readily and almost quantitatively cross-linked, indicating an intimate association between both polypeptides. When a cleavable cross-linker was used, the cross-linking products could be partially dissociated into the individual subunits. As a control, a monomeric protein (chicken lysozyme) was not cross-linked and remained monomeric under the same conditions (Figure 1B).

Having established that glucosidase α and β subunits appear to associate intimately (Figure 1), we analyzed the

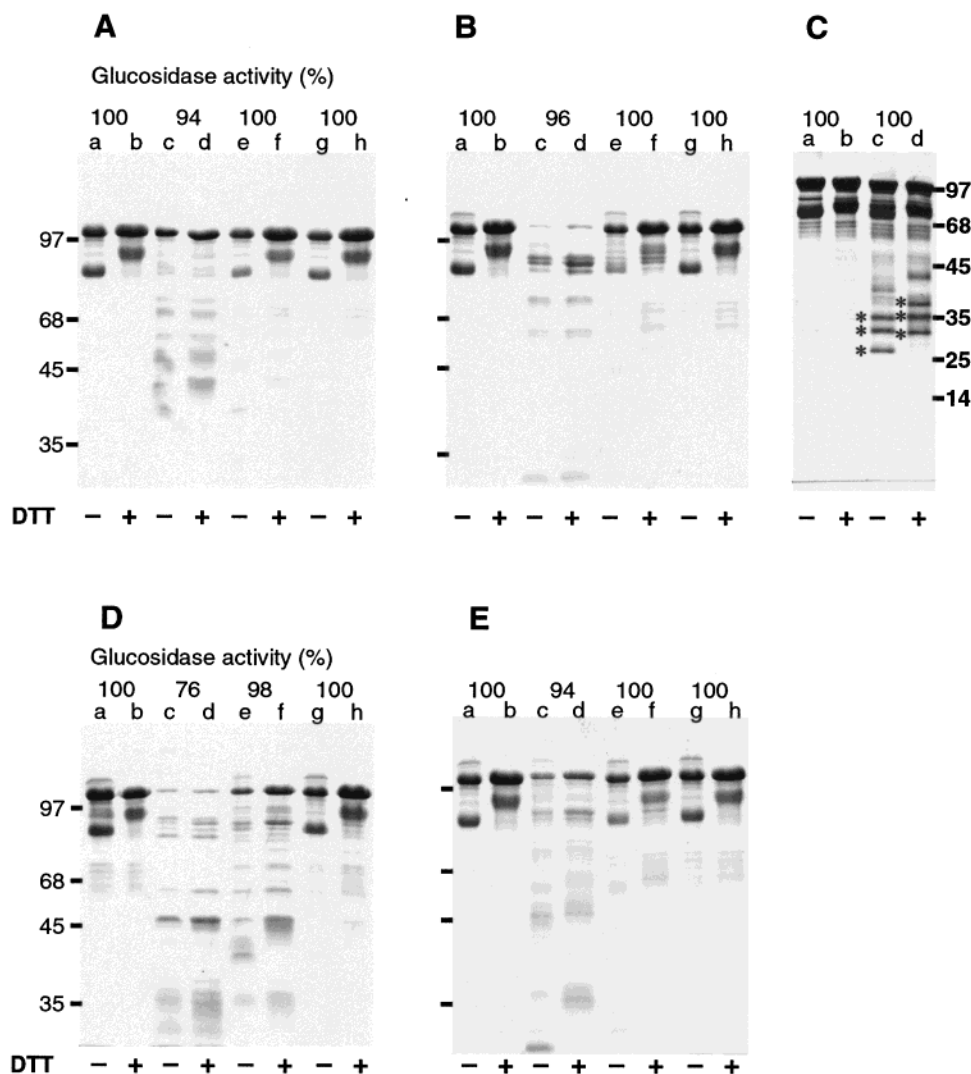


FIGURE 4: β subunit is not required for glucosidase activity in vitro. Rat liver glucosidase II was digested with chymotrypsin (A); endo Glu-C (B and C); endo Lys-C (D); and subtilysin (E). In all lanes labeled a and b, no protease was added. Lanes c and d, e and f, or g and h represent increasing glucosidase/tease ratios (1:50–1:5000). After 1 h at 25 °C, digestions were analyzed by 12.5% SDS–PAGE. In panel C, lanes c and d, glucosidase II (2 μ g) was incubated with endo Glu-C (1:20 000 ratio) for 30 min on ice, and the reaction was analyzed by 15% SDS–PAGE. The six fragments marked with an asterisk (*) have the same N-terminus corresponding to the N-terminus of the mature β subunit (VEVKRP). Numbered marks at the sides of the gels denote the mass of the molecular markers in kilodaltons.

behavior of the complex in solution and estimated its size. The molecular mass for the complex was investigated using sedimentation equilibrium. The enzyme was found to exist predominantly (>90%) as a well-defined complex of 161 kDa. Such value is in optimal agreement with the estimated molecular mass calculated from the amino acid composition of a hypothetical 1:1 complex of mature α and β subunits (161.3 kDa). Although the calculation does not take into account N-glycosylation or other posttranslational modifications, it still represents the most likely arrangement of α and β subunits that would result in a complex of 161 kDa. The behavior of the complex in these experiments did not vary at concentrations ranging from 0.1 to 1.0 mg/mL. Results from a typical sedimentation equilibrium profile are shown in Figure 2. A global nonlinear least-squares analysis of nine data sets collected at three initial concentrations and three speeds was carried out using NONLIN. The simplest model that adequately describes the data contains two species with molecular weights corresponding to a monomeric $\alpha_1\beta_1$ complex as well as a small amount (<10%) of higher order

oligomers of this $\alpha_1\beta_1$ complex, best described as tetramers (Figure 2). The formal relation is given by

$$c_i = c_{\text{ref}} \exp[\sigma(\xi_i - \xi_{\text{ref}})] + c_{n,\text{ref}} \exp[n\sigma(\xi_i - \xi_{\text{ref}})] + \text{base} \quad (1)$$

where $\sigma = M(1 - \bar{v}\rho)\omega^2/RT$; $\xi = r^2/2$; M is the molecular mass; \bar{v} is the partial specific volume (mL g^{-1}); ρ is the solvent density (g mL^{-1}); ω is the angular velocity (rad s^{-1}); R is the universal gas constant; T is the absolute temperature; c_i and c_{ref} are the absorbance values at positions r_i and r_{ref} , respectively; n is the oligomeric state of the higher order oligomer; and base is a baseline term for nonsedimenting material.

The value for σ was calculated from the calculated amino acid composition using SEDNTERP and was set to correspond to the monomeric $\alpha_1\beta_1$ complex protein. The fit was judged to be optimal by examination of the residuals (small and random) and minimization of the variance (6.96×10^{-3} with 909 DF).

The behavior of the $\alpha_1\beta_1$ complex in solution was also studied by sedimentation velocity experiments in analytical ultracentrifugation (Figure 3). The experimentally observed sedimentation coefficient for the $\alpha_1\beta_1$ complex was 6.3 ± 0.1 S (Figure 3A), which corresponds to a $s_{20,w}$ of 6.4 S. There was no observed concentration dependence over the concentration range studied (0.1–1.0 mg/mL). Using the Svedberg equation, we calculated a frictional coefficient, $f_{20,w}$, of 11.2×10^8 g s⁻¹. Straightforward interpretation of the frictional coefficient to estimate the axial ratio of the $\alpha_1\beta_1$ complex suggests an overall structure that is highly nonglobular. Models employing either a prolate or an oblate ellipsoid of revolution are shown in Figure 3B.

The hydrodynamic experiments revealed that the $\alpha_1\beta_1$ complex was stable and well-defined. We could not find conditions to disassemble the complex preserving glucosidase activity, to isolate the individual subunits to study their interaction, or to evaluate the requirements of the β chain for activity. We therefore used limited proteolysis to identify domains within the complex. We found that, even under mild proteolytic conditions, the β subunit was extremely sensitive to proteases (Figure 4). Using small amounts of Glu-C endoproteinase on ice, we could detect three fragments representing intermediates in the degradation of the β subunit (highlighted with asterisks in Figure 4C). Their increased mobility under nonreducing conditions indicated that they contained intramolecular disulfide bonds. These fragments have the same N-term sequence (VEVKRP), corresponding to the N-term of the mature β subunit.

The β Subunit Is Not Required for Glucosidase II Activity In Vitro. Under several different proteolytic conditions, the β subunit was consistently more readily degraded than the α subunit (Figure 4). Preincubation of the samples with CaCl₂ or EDTA or their inclusion in the incubations did not affect the proteolysis pattern. The higher resistance to proteolysis of the α subunit allowed us to prepare samples in which the β subunit was partially cleaved (Figure 4C, lanes c and d) or completely degraded (Figure 4A, lanes c and d; Figure 4B,D, lanes e and f), while the remaining α subunit retained up to 100% activity. Furthermore, limited digestion with trypsin resulted in a fully active fragment of 70 kDa (Figure 5). This fragment coeluted in gel filtration with a standard of 68 kDa, indicating that it was monomeric and globular. The N-terminal sequence of this fragment (MMDYLQGSGETPQTDV) indicates that it begins at Met346 of the α subunit, mapping this C-term globular domain to the region of the α subunit that shows the highest homology to other glucosidases.

DISCUSSION

We demonstrate here that rat liver glucosidase II is a defined complex of one α subunit and one β subunit. With a molecular mass of 161 kDa and a s value of 6.3 S, the $\alpha_1\beta_1$ complex appears to have a highly nonglobular shape. Alternatively, the $\alpha_1\beta_1$ complex could have a less extended shape but possess domains that are highly flexible and create drag in transport experiments. These interpretations are consistent with the elution pattern of glucosidase II from gel filtration columns at the position expected for a 450-kDa globular protein (7).

In agreement with its proposed highly extended conformation, the $\alpha_1\beta_1$ complex was very sensitive to proteolysis. The

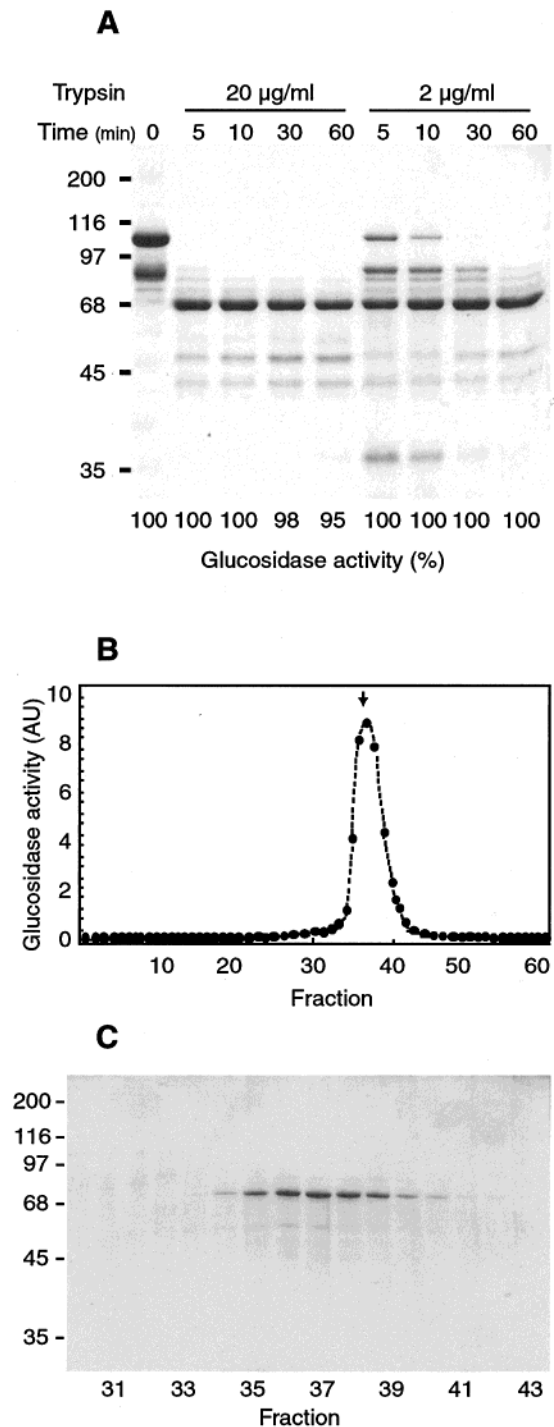


FIGURE 5: Identification of the catalytic domain in the α subunit of glucosidase II. (A) Rat liver glucosidase II was digested with trypsin, and at the indicated time points, aliquots were removed and analyzed for glucosidase activity and by SDS-PAGE stained with Coomassie blue. (B) Rat liver glucosidase II was digested with trypsin, and the digested product was fractionated on a Superose12 column. Fractions were collected and assayed for glucosidase activity. The arrow indicates the elution of a 68 kDa standard (bovine serum albumin). (C) Aliquots (5 μ L) of the samples obtained in B were analyzed by SDS-PAGE and stained with Coomassie blue. The N-terminal amino acid sequence of the 68-kDa fragment found across the peak of glucosidase activity was MMDYLQGSGETPQTDV. Numbered marks at the sides of the gels denote the mass of the molecular markers in kilodaltons.

most stable domain in the $\alpha_1\beta_1$ complex corresponds to the C-term, two-thirds of the α subunit, comprising a fully active

catalytic core of 70 kDa that is globular and monomeric. Interestingly, this fragment maps to the region of its primary structure with the highest homology to other glycosidases (7), some of which have been clearly shown to fold into a globular structure (23, 24). Sites of alternative splicing have been identified in the α subunit, but they are not located in the stable catalytic core (11, 12, 25). Alternatively, spliced transcripts have also been found for the β subunit (11, 12, 25), but the function of the different isoforms is unknown.

The identification of a globular domain in the α subunit would imply that the other portions of the complex (the β subunit and the N-terminal third of the α subunit) adopt an extended conformation, contributing to the asymmetric shape of the complete oligomer and resulting in their higher susceptibility to proteolysis. In support for this, the N-terminal third of the α subunit was systematically more sensitive to proteolysis than the rest of the α subunit, and the β subunit was the most sensitive to proteolytic degradation. Detection of an N-terminal fragment of the β subunit containing S–S bonds suggests that the N-term, cysteine-rich region does not make S–S bonds with the Cys-rich portion at the C-term. The β subunit thus seems to adopt an extended conformation that may contain successive domains along the primary sequence, suggested by clearly predictable secondary structure elements. The portion containing the poly-Glu stretch flanked by Pro-rich regions may act as a spacer between the N-terminal and the C-terminal domains of the molecule. It is still possible that the relaxed structure observed in our experiments is due to the loss during the purification procedure of one or more protein(s) that might normally bind to the β subunit.

The marked protease sensitivity of the β subunit allowed us to confirm that it is not required for glucosidase activity of the $\alpha_1\beta_1$ complex *in vitro*. This finding may explain why the β subunit has not been detected in other studies of glucosidase II, where it may have been degraded during the isolation, and the more stable α subunit maintained glucosidase activity (16–18, 26).

We demonstrate that the isolated globular catalytic core of the α subunit retains the same glucosidase II activity as in the intact $\alpha_1\beta_1$ complex. Incorporation of this catalytic core into a markedly nonglobular $\alpha_1\beta_1$ complex is probably required to bridge glucosidase II activity to other components in the ER. These interactions seem to be required for proper localization or assembly of the α subunit, since the β subunit is required for glucosidase II activity in living cells (14). The mechanism by which it affects glucosidase II activity in the ER remains to be established, but it most likely retains the $\alpha_1\beta_1$ complex in the ER through its C-terminal HDEL sequence. It could also be required for proper folding of the α subunit or for association with other ER components. Interestingly, homologues of the β subunit cannot be identified in *Saccharomyces cerevisiae*. If it is indeed absent in this organism, then the α subunit may have other requirements for assembly or ER localization, or perhaps,

some yet unidentified protein replaces the β subunit in this organism. The catalytic domain is the most conserved region between the *S. cerevisiae* α subunit and other α subunits and glycosidases, while the N-terminal portion differs the most. This N-term domain of the α subunit is also where alternative splicing occurs in mammalian cells and could be engaged in protein–protein interactions with the β subunit or other proteins in the lumen of the ER. However, the region(s) of association between both subunits remains to be established. Understanding the emerging complexity of the deglycosylation machinery will provide further insight into the mechanisms of glycoprotein biogenesis in the ER.

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