Lysosomal Acid α-Glucosidase Consists of Four Different Peptides Processed from a Single Chain Precursor*

Rodney J. Moreland‡, Xiaoying Jin§, X. Kate Zhang§, Roger W. Decker‡, Karen L. Albee§, Karen L. Lee§, Robert D. Cauthron‡, Kevin Brewer‡, Tim Edmunds§, and William M. Canfield‡

From the §Genzyme Corporation, Oklahoma City, Oklahoma 73104 and the ¶Genzyme Corporation, Framingham, Massachusetts 01701

Pompe’s disease is caused by a deficiency of the lysosomal enzyme α-glucosidase (GAA). GAA is synthesized as a 110-kDa precursor containing N-linked carbohydrates modified with mannose 6-phosphate groups. Following trafficking to the lysosome, presumably via the mannose 6-phosphate receptor, the 110-kDa precursor undergoes a series of complex proteolytic and N-glycan processing events, yielding major species of 76 and 70 kDa. During a detailed characterization of human placental and recombinant human GAA, we found that the peptides released during proteolytic processing remained tightly associated with the major species. The 76-kDa form (amino acids aa 122–782) of GAA is associated with peptides of 3.9 kDa (aa 78–113) and 19.4 kDa (aa 792–952). The 70-kDa form (aa 204–782) contains the 3.9- and 19.4-kDa peptide species as well as a 10.3-kDa species (aa 122–199). A similar set of proteolytic fragments has been identified in hamster GAA, suggesting that the multicomponent character is a general phenomenon. Rabbit anti-peptide antibodies have been generated against sequences in the proteolytic fragments and used to demonstrate the time course of uptake and processing of the recombinant GAA precursor in Pompe’s disease fibroblasts. The results indicate that the observed fragments are produced intracellularly in the lysosome and not as a result of nonspecific proteolysis during purification. These data demonstrate that the mature forms of GAA characterized by polypeptides of 76 or 70 kDa are in fact larger molecular mass multicomponent enzyme complexes.

Lysozymal acid α-glucosidase (GAA; EC 3.2.1.3) is an exo-1,4- and -1,6-α-glucosidase that hydrolyzes glycogen to glucose. The cDNA for GAA encodes a protein of 952 amino acids with a predicted molecular mass of 105 kDa (1). The newly synthesized precursor has an amino-terminal signal peptide for cotranslational transport into the lumen of the endoplasmic rexisized acid α-glucosidase (GAA1; EC 3.2.1.3) is an exo-1,4- and -1,6-α-glucosidase that hydrolyzes glycogen to glucose. The cDNA for GAA encodes a protein of 952 amino acids with a predicted molecular mass of 105 kDa (1). The newly synthesized precursor has an amino-terminal signal peptide for cotranslational transport into the lumen of the endoplasmic reticulum, where it is N-glycosylated at seven glycosylation sites, resulting in a glycosylated precursor with an apparent molecular mass of 110 kDa.

The intracellular processing of GAA has been investigated previously (2, 3). It was proposed that, after transport through the Golgi complex and targeting to the endosome/lysosome, the 110-kDa precursor is proteolytically processed at the amino terminus, resulting in a 95-kDa intermediate with a sequence beginning at amino acid 122. Prior to this study, the 95-kDa intermediate was proposed to be proteolytically processed to a 76-kDa form, which was believed to occur between amino acids 816 and 881 (3). The 76-kDa form is then proteolytically processed at the amino terminus to amino acid 204 to give the 70-kDa mature form (3). The nomenclature used for the processed forms of GAA is based on apparent molecular mass as determined by SDS-PAGE.

The identities of the proteases involved in the maturation of GAA have never been established. GAA has been purified from many different tissues such as bovine testis (4), rat liver (5), pig liver (6), human liver (7), rabbit muscle (8), human heart (9), human urine (10), and human placenta (2, 11). The predominant species observed are the 76- and 70-kDa mature forms. The fate of the cleaved fragments is unknown. Several reports have noted the presence of small polypeptides in highly purified preparations of the 76- and 70-kDa forms (2, 10, 14). It was suggested that the small polypeptides might be contaminants, degradation products, or a previously described GAA activation protein (11).

Proteolytic processing appears to be required for optimal activity toward the natural substrate glycogen. There is a 7–10-fold increase in the affinity of the 76/70-kDa species for glycogen compared with the 110-kDa precursor (3, 17). In addition to the proteolytic maturation of the GAA peptide backbone, there is extensive processing of the carbohydrate chains. GAA is targeted to the lysosomes by the mannose 6-phosphate receptor, but analysis of the carbohydrate chains from purified 76/70-kDa GAA from human placenta revealed the absence of mannose 6-phosphate and additional carbohydrate processing (18).

A deficiency of acid α-glucosidase causes Pompe’s disease, which results in the accumulation of glycogen in lysosomes. Pompe’s disease is an autosomal recessive disorder that varies from a fatal infantile form to a more slowly debilitating adult-onset form (reviewed in Ref. 19). Efforts are under way to develop enzyme replacement therapy for Pompe’s disease. Several similar but distinct transgenic (2, 3) or recombinant (17, 18) GAA preparations have been developed for this purpose. Several reports have demonstrated that enzyme replacement therapy using the 110-kDa precursor form of GAA degrades lysosomal glycogen in cultured Pompe’s disease fibroblasts and GAA knockout mice (2, 3, 18). Although the 110-kDa form of

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‡ To whom correspondence should be addressed: Genzyme Corp., 800 Research Parkway, Suite 200, Oklahoma City, Oklahoma 73104. Tel.: 405-271-8144; Fax: 405-271-8189; E-mail: william.canfield@genzyme.com.

1 The abbreviations used are: GAA, acid α-glucosidase; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; HPLC, high pressure liquid chromatography; rhGAA, recombinant human acid α-glucosidase; CHO, Chinese hamster ovary; HP-rhGAA, highly phosphorylated recombinant human acid α-glucosidase; PVDF, polyvinylidene difluoride; MALDI-TOF-MS, matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry; LC/MS, liquid chromatography/mass spectrometry; MS/MS, tandem mass spectrometry; CAPS, 3-(cyclohexylamino)propanesulfonic acid; aa, amino acids.
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GAA has been administered therapeutically, it is the mature forms that are found in the lysosome and that are most active toward the glycogen substrate.

In this study, we demonstrate that proteolytically processed GAA purified from human placenta consists of four different peptides processed from a single chain precursor. We demonstrate that the recombinant 110-kDa GAA precursor is internalized by Pompe’s disease fibroblasts via the mannose 6-phosphate receptor and undergoes proteolytic cleavage during which several of the cleaved fragments remain associated.

**EXPERIMENTAL PROCEDURES**

**Materials**

Concanavalin A, DEAE-Sepharose FF, and Superdex 200 prep grade were obtained from Amersham Biosciences. α-Methyl glucoside, benzamidine, methionine sulfoximine, and 4-methylumbelliferyl α-D-glucoside were obtained from Sigma. Other chemicals were reagent grade or better and were from standard suppliers. SDS-polyacrylamide gels were obtained from Invitrogen. Antibodies were made by Washington Biotechnology (Baltimore, MD). Roller bottles were obtained from Corning. Dulbecco’s modified Eagle’s medium and fetal bovine serum were obtained from JRH Biosciences. Dialyzed phosphate-buffered saline was obtained from HyClone Laboratories.

**Acid α-Glucosidase Activity and Protein Assay**

Acid α-glucosidase was assayed fluorometrically in a microtiter plate using 4-methylumbelliferyl α-D-glucoside as a substrate in 25 mM sodium acetate (pH 4.8) (10). Protein concentration was estimated by absorbance at 280 nm assuming ε\text{max} = 10 or using the micro BCA assay (Pierce) standardized with bovine serum albumin (20).

**SDS-PAGE**

Reduced and nonreduced samples and molecular mass markers (Amersham Biosciences) were applied to a 4–20 or 10% Tris/glycine/SDS-polyacrylamide gel (Invitrogen). Electrophoresis was performed at 150 V for 1.5 h, and proteins were visualized with either Coomassie Blue or silver stain (21).

**Buffers**

Buffer A contained 25 mM Tris-HCl (pH 6.5), 2 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 50 mM NaCl, and 0.25% Triton X-100.

Buffer B contained 20 mM Tris-HCl (pH 6.5), 1 mM CaCl\text{2}, 1 mM MnCl\text{2}, and 500 mM NaCl.

Buffer C contained 20 mM BisTris-HCl (pH 5.5).

Buffer D contained 20 mM BisTris-HCl (pH 5.5) and 500 mM NaCl.

Buffer E contained 3 mM (NH\text{4})\text{2}SO\text{4} and 75 mM Tris-HCl (pH 6.5).

Buffer F contained 1 M (NH\text{4})\text{2}SO\text{4} and 250 mM NaH\text{2}PO\text{4} (pH 6.5).

Buffer G contained 50 mM NaH\text{2}PO\text{4} (pH 6.5) and 20 mM NaCl.

Buffer H contained 40 mM NaH\text{2}PO\text{4} (pH 6.5), 39 mM glycine, 20% MeOH, and 0.04% SDS.

Buffer I contained 50 mM NaH\text{2}PO\text{4} (pH 6.5) and 20 mM NaCl.

Buffer J contained 20 mM NaH\text{2}PO\text{4} (pH 6.5).

**Cell Lines**

CHO-K1 cells were obtained from American Type Culture Collection (Manassas, VA). The Pompe’s disease fibroblast cell line GM00248 was obtained from Coriell Cell Repositories (Camden, NJ). This cell line is homozygous for the common African-American R854X mutation.

**Purification of Hamster Acid α-Glucosidase from Chinese Hamster Ovary Cells**

**Step 1: Harvesting Cells and Cell Lysis**—CHO-K1 cells were expanded to 300 1700 cm\textsuperscript{2} roller bottles in medium containing Dulbecco’s modified Eagle’s medium/1% glutamine synthase supplement, 10% dialyzed fetal bovine serum, 25 μM methionine sulfoximine, and 5% CO\textsubscript{2} at 37 °C. Each bottle was rinsed with 40 ml of dialyzed phosphate-buffered saline and then rolled for 30 min at 37 °C in the presence of 40 ml of buffer A. The cell lysate was clarified with a 10-inch 0.2-μm Opticap filter (Millipore Corp.) and stored at −20 °C.

**Step 2: Concanavalin A**—The clarified cell lysate was adjusted to 1 mM MnCl\text{2} and 1 mM CaCl\text{2} and applied to a concanavalin A column (2.6 × 20 cm) equilibrated with buffer B at 9 ml/min. The column was washed with 10 column volumes of buffer B and step-eluted with buffer B containing 0.25 M α-methyl glucoside. Three 0.25-litre fractions were collected and pooled.

**Step 3: Phenyl-Sepharose 6 FF**—The clarified cell lysate was adjusted to 1 mM (NH\text{4})\text{2}SO\text{4} with buffer E and applied to a phenyl-Sepharose 6 FF column (1.6 × 20 cm) equilibrated with buffer F at 6.5 ml/min. After washing the column with 5 column volumes of buffer F, the column was washed with buffer F containing 0.25 M (NH\text{4})\text{2}SO\text{4}. Placentally purified enzyme was eluted with a 5-column volume gradient from 0.25 to 0 M (NH\text{4})\text{2}SO\text{4} buffer F. Fractions of 15 ml were collected and assayed for acid α-glucosidase activity and protein.

**Step 4: Superose 6—A peak fraction from the Superdex 200 elution was applied to a Superose 6 column (10 × 30 cm) equilibrated with buffer G at 2 ml/min. Fractions of 2 ml were collected and assayed for acid α-glucosidase activity and protein.

**Recombinant Human GAA (rhGAA) Precursor and Intermediate**

The rhGAA precursor and intermediate were produced using Chinese hamster ovary cell (CHO) cells transfected with a vector containing a full-length human GAA cDNA following the method described previously (22). rhGAA was purified from the CHO-conditioned medium as follows. The CHO cell culture harvest was clarified by sequential direct flow filtration using a polyethersulfone pre-filter, followed by a Whatman Polyvac 75TC 0.2-μm PES membrane filter. The clarified harvest...
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Preparation of Recombinant Human GlcNAc-phosphotransferase

A detailed description of the preparation of GlcNAc-phosphotransferase will be reported elsewhere. Briefly, a production cell line secreting recombinant soluble human GlcNAc-phosphotransferase was generated by transfection of CHO cells with a "double gene" expression vector in PEE141/pPEE6.1 (Lonza Biologics, Portland, NH) containing modified human GlcNAc-phosphotransferase α/β-subunit sequences and the native γ-subunit sequence. The α/β-subunit cDNA contained four modifications of the native sequence. First, the amino-terminal signal anchor sequence was replaced with a signal peptide derived from the mouse immunoglobulin light chain. Second, the carboxyl-terminal transmembrane sequence was deleted by truncation of the 110-kDa precursor by Superdex 200 affinity chromatography. The uncovering enzyme removes the GlcNAc to give glycans containing terminal mannose were phosphorylated by treating the 110-, 95-, and 76-kDa forms was purified from conditioned medium, and the HP-rhGAA was used in a 1:5 ratio with sodium phosphate buffer (pH 6.5).

Purification of Highly Phosphorylated rhGAA (HP-rhGAA)

A detailed method for the production of HP-rhGAA will be described elsewhere. Briefly, rhGAA was expressed in CHO cells grown in the presence of kifunensine at 1 mg/liter. Kifunensine selectively inhibits the UDP-GlcNAc:polypeptide N-acetylglucosaminyltransferase to GAA ratio of 1:10 (w/w). UDP-GlcNAc was added to the protein sample at a ratio of 1:50 (w/w). After an 18-h incubation at 37 °C, the digestion was quenched with 0.1% trifluoroacetic acid and stored at −20 °C for future analysis.

Matrix-assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) Analysis of Reduced and Nonreduced Protein Samples

Protein samples were mixed at a 1:1 ratio with saturated sinapinic acid matrix solution (50% of 0.1% trifluoroacetic acid and H2O and 50% acetonitrile), and 1 μl of the mixture was applied to the sample target. The data were acquired in positive linear mode on a Voyager DE PRO MALDI-TOF mass spectrometer (Applied Biosystems). Additionally, and doubly charged molecular ions of bovine serum albumin (Sigma) were used to calibrate for the mass range of 20–140 kDa. The singly charged mass spectra of intact native GAA were acquired, and Figure 3 (Applied Biosystems) was used to calibrate for the mass range of 2–20 kDa.

Capillary Liquid Chromatography/Mass Spectrometry (LC/MS) Analysis of Reduced and Nonreduced Protein Samples

Capillary LC/MS was performed on a QSTAR QQ-TOF mass spectrometer (Applied Biosystems) interfaced with an Ultimate capillary LC system (LC Packings). Protein separations were performed on a Vydac C4 reverse-phase column (320 μm × 50 cm; Micromechanical Scientific) with a mobile phase of 1% formic acid in water (A) and acetonitrile (B). The flow rate was 4 μl/min. The mass spectra were acquired in the positive mode at a range of m/z 800–3000. Protein reduction was performed by adding 1 μl of 10% β-mercaptoethanol to every 10 μl of protein sample. The samples were sonicated for 25 min and heated at 100 °C for 1 min before MS analysis.

Capillary LC/Tandem Mass Spectrometry (MS/MS) Peptide Mapping of Protein Tryptic Digests

On-line capillary LC/MS/MS was performed on both Esquire LC (Bruker) and QSTAR QQ-TOF systems. Proteins were separated on a Vydac C18 column (320 μm × 15 cm; Micromechanical Scientific) using the chromatography conditions described above. On both instruments, the two ESI intensive peaks were merged into the for on-line MS/MS.

Amino-terminal Microsequencing

Samples of reduced and nonreduced rhGAA and placental GAA were applied to a 4–20% or 6% Tris/glycine/SDS-polyacrylamide gel (Invitrogen). Electrophoresis was performed at 150 V for 1.2 h. The proteins were transferred to a PVDF membrane using 10 mm CAPS (pH 11.0). Transfer was performed for 1 h at 100 V. Protein bands were visualized with SuperSignal detection kit (Pierce).
with Coomassie Blue. Fifteen cycles of automated Edman chemistry were performed on protein bands excised from PVDF electroblots. Amino-terminal sequencing was performed with the Model 492 Procise Protein Sequencer (Applied Biosystems) using the preprogrammed pulsed-liquid PVDF method.

**Affinity Chromatography of GAA**

Twenty μg of purified human placental GAA and rhGAA was analyzed by affinity chromatography using a Superdex 75 HR 10/30 column (Amersham Biosciences). GAA is retarded because of its affinity for the dextran backbone of the Superdex matrix. The column was equilibrated with 20 mM NaH₂PO₄ (pH 6.0) and 200 mM NaCl at a flow rate of 0.5 ml/min. Peaks were detected at 215 and 280 nm with a Model 1100 HPLC system equipped with a diode array detector (Agilent). The column was calibrated with Bio-Rad gel filtration standards prior to sample analysis. Fractions were collected at 2-min intervals and analyzed by SDS-PAGE.

**Fibroblast Uptake of HP-rhGAA**

For each time point, ~0.5 million Pompe’s disease fibroblasts (GM00248) in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum were incubated with 50 nM highly phosphorylated GAA prepared as described above. At designated time points, the cells were removed and washed five times with phosphate-buffered saline and stored at ~80 °C. At 24 h, the cells were washed, and fresh medium that did not contain GAA was added. After the final time point, all cell pellets were thawed and lysed simultaneously with 0.25% Triton X-100. Cellular debris was pelleted, and Western blot analysis was performed on supernatants from each time point with several different antibodies to GAA. The blot was developed as described under “Western blot analysis.”

**RESULTS**

**Placental GAA Contains Low Molecular Mass Polypeptides Derived from GAA—**GAA was purified from human placenta by concanavalin A-Sepharose, phenyl-Sepharose, and Superdex 200 chromatography. The final step was affinity chromatography, which takes advantage of the retardation of GAA because of its affinity for the dextran backbone of the Superdex matrix (11, 14, 25). Analysis of purified human placental GAA by silver staining/SDS-PAGE demonstrated two consistent low molecular mass polypeptides with apparent masses of 19 and 10 kDa in addition to the expected 76- and 70-kDa bands (data not shown). Amino-terminal sequence analysis of PVDF-transferred protein indicated that the amino termini of the 19- and 10-kDa forms are APREPAHISEQG and MGGPXXFPFP, respectively. A database search using the BLASTP algorithm (24) indicated that the sequences of the 19- and 10-kDa polypeptides were from human GAA and correspond to amino acids 792–803 and 122–131, respectively.

Since processed GAA is significantly retarded on Superdex 200, it was uncertain if the apparent coelution of the 19- and 10-kDa polypeptides with the 76- and 70-kDa proteins indicated a physical association. To provide evidence that the 19- and 10-kDa polypeptides were physically associated with the 76- and 70-kDa bands, fractions were collected at 2-min intervals and analyzed by SDS-PAGE.

**Characterization of the rhGAA Precursor and Processing Intermediates**—It has been shown previously that recombinant CHO cells can be engineered to secrete the 110-kDa precursor form of human GAA into tissue culture medium (25). In addition to the 110-kDa precursor form, recombinant CHO cells also release proteolytically processed forms of GAA into the medium by an undetermined mechanism. In Fig. 2A, the rhGAA precursor (lane 2) and processing intermediates (lane 3) and mature GAA from placenta (lane 4) are compared by SDS-PAGE. The intermediate fraction contains bands of ~108, 95, and 76 kDa. The availability of these intermediates provided a unique opportunity to study the processing/activation of GAA. The preparations shown in Fig. 2A were analyzed by affinity chromatography on Superdex 200 (Fig. 2B). In each case, virtually all the protein was derived from GAA. Inspection of the chromatograms demonstrated that the 110-kDa precursor eluted earlier than placenta-derived 76/70-kDa mature GAA and that the intermediate preparation contained both early and late eluting material. SDS-PAGE analysis of the late eluting material revealed that the 76- and 70-kDa forms were not separated by affinity chromatography (data not shown). The later elution of the processed forms suggests a higher affinity for the matrix, which is consistent with previous reports that the 76/70-kDa species has a Kₘ for glycogen that is 7–10-fold lower than that of the 110-kDa precursor (3, 17).

**Characterization of the rhGAA Precursor and Processing Intermediates by Mass Spectroscopy and Amino-terminal Micro-
To fully characterize GAA processing, we utilized a combination of MALDI-TOF-MS, amino-terminal microsequencing, capillary LC/MS, and capillary LC/MS/MS of tryptic peptide maps. This allowed us to determine the amino and carboxyl termini for each polypeptide and the structure of the N-glycans. The results of these analyses are summarized in Table I.

When the recombinant GAA precursor was examined by MALDI-TOF-MS, a molecular mass of 111,600 Da was determined. Amino-terminal microsequencing identified amino acid 57 as the amino terminus. Tryptic digestion followed by reverse-phase HPLC and peptide identification by mass spectrometry identified amino acid 952 as the carboxyl terminus. The glycosylation is heterogeneous and estimated at ~2000 Da/glycan. These results for the precursor (mass of 111,600 Da) are in reasonable agreement with the calculated molecular mass of the polypeptide and carbohydrate of 113,372 Da.

When the processing intermediates containing predominantly 95- and 76-kDa species upon reducing SDS-PAGE (Fig. 2A, lane 3) were analyzed unreduced by MALDI-TOF-MS, major species with masses of ~103.5 and ~83.6 kDa were observed. After reduction, masses of ~99.8 and ~79.7 kDa were observed, indicating a consistent mass shift of ~3.9 kDa, consistent with an interchain disulfide bond.

Analysis of human placental GAA by reducing SDS-PAGE (Fig. 1) identified polypeptides with apparent molecular masses of ~95, 76, 70, 19, and 10 kDa. MALDI-TOF-MS analysis of reduced placental GAA determined that the corresponding masses were ~99.8, ~79.7, 69.3, 19.4, and 10.3 kDa. MALDI-TOF-MS analysis of nonreduced placental GAA re-
vealed polypeptides with masses of ~103.5, ~83.6, 69.3, 19.4, and 14.3 kDa. These data show the ~103.5- to ~83.6- and 14.3-kDa polypeptides all shifted by ~3.9 kDa after disulfide bond reduction, again suggesting the presence of an interchain di-sulfide bond. This experiment was also repeated by LC/MS using a QSTAR QQ-TOF mass spectrometer. The ~3.9-kDa mass shift seen in all reduced samples correlated with a new peak in the spectra with a mass of 3927 Da.

The GAA sequences found in each of the identified polypeptides were determined by a combination of microsequencing, tryptic digestion, capillary LC/MS, and LC/MS/MS. The 3.9-kDa polypeptide with a determined mass of 3927 Da corresponded to amino acids 78–113. The 10.3-kDa polypeptide was identified as amino acids 122–199. The 70-kDa polypeptide corresponds to amino acids 204–782. The 19.4-kDa polypeptide was identified as amino acids 792–952. These results are summarized in Table I.

**Modification of N-Glycans during GAA Maturation**—The GAA precursor contains seven consensus sites for N-glycosylation that are all utilized. In recombinant GAA, on average, these sites are occupied by five to six complex-type glycans and one to two high mannose-type glycans. In contrast, GAA expressed in human placenta contains predominantly high mannose-type glycans (18). In either case, the newly synthesized glycans each contain ~9–11 hexoses/hexosamines and have an average molecular mass of ~2000 Da.

By mass spectroscopy, the recombinant precursor contains ~14,000 Da of carbohydrate, indicating an average of ~10 monosaccharides/glycan (Table I). The 95-kDa intermediate contains 7300 Da of carbohydrate at these same seven glycosylation sites, indicating that the glycans have been truncated to an average of approximately five monosaccharides, consistent with an average core structure composed of two GlcNAc and three mannose residues, which can be derived from either complex-type or high mannose-type glycans. These results suggest that the conversion of the 110-kDa precursor to the 95-kDa intermediate has occurred in a subcellular compartment containing a variety of glycosidases capable of cleaving a complex-type glycan. This pattern of finding only highly truncated glycans on the processed GAA is repeated for all the components of the 76- and 70-kDa complexes (Table I).

| Apparent molecular mass by SDS-PAGE (reducing) | Polyepitopes present (amino acids) | No. of glycosylation sites | Observed carbohydrate structures by MS | Molecular mass of polypeptide | Theoretical molecular mass of polypeptide | Calculated molecular mass of polypeptide + carbohydrate | Observed molecular mass of polypeptide + carbohydrate by MS | Calculated molecular mass of complex | Observed molecular mass of complex by MS (nondenaturing/nondecreasing) | Source |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **kDa** | **Dn** | **Dn** | **Dn** | **Dn** | **Dn** | **Dn** | **Dn** | **Dn** | **Dn** | **Dn** | **Dn** |
| 110 | 57–952 | 7 | Heterogeneous glycoforms | ~14,000 | 99,372 | ~113,372 | 111,600 | ~113,372 | 111,600 | Recombinant |
| 95 | 78–113 | 0 | None | None | None | None | None | None | None | Recombinant |
| 76 | 122–952 | 5 | Heterogeneous glycoforms | ~6000 | 74,277 | ~80,277 | 78,700 | ~80,277 | 78,700 | Recombinant |
| 70 | 78–113 | 0 | None | None | None | None | None | None | None | Recombinant |
| 72 | 122–199 | 1 | 2 HexNac, 5 Hex | 1216 | 9177 | 10,393 | 10,392 | 10,393 | 10,392 | Recombinant |
| 70 | 204–782 | 4 | 8 HexNac, 18 Hex, 1 Fuc | 4866 | 84,639 | 89,325 | 89,328 | 89,328 | 89,328 | Recombinant |
| 70 | 792–952 | 2 | 4 HexNac, 6 Hex, 2 Fuc | 2078 | 17,370 | 19,448 | 19,447 | 19,447 | 19,447 | Recombinant |

a ND, not determined.

Identification of Subunits with Anti-peptide Antibodies—To independently evaluate the model for GAA maturation derived from mass spectrometry, polyclonal, monoclonal, and anti-peptide antibodies were generated against peptide sequences QIIGASRPGPRDAQAHPGR (amino acids 57–74), VPTQCDVPPNSRFDACP (amino acids 78–94), and IKDPANRYYEVPLETRV (amino acids 183–200) as described under “Experimental Procedures.” The resulting affinity-purified antisera are referred to as anti-GAA-(57–74), anti-GAA-(78–94), and anti-GAA-(183–200), respectively. Additionally, a goat polyclonal antibody to placental GAA (goat anti-GAA) and a monoclonal antibody to the 70-kDa polypeptide (GAA1) were generated as described under “Experimental Procedures.”

As shown in Fig. 3, Western blot analysis was performed with a variety of antibodies on a blot containing the 110-kDa HP-rhGAA precursor (P), an intermediate preparation (I) of 95/76-kDa forms from the purification of HP-rhGAA, and the 95/76-kDa mature species (M) purified from human placenta. HP-rhGAA is a highly phosphorylated form of rhGAA derived from in vitro phosphorylation as described under “Experimental Procedures.” Monoclonal antibody GAA1 recognized the 110-, 95-, 76-, and 70-kDa species (Fig. 3A, lanes 1–3). The blot was then probed again with goat anti-GAA antibody, which demonstrated the presence of the 19-kDa fragment (Fig. 3A, lanes 4–6). The 19.4-kDa fragment exists as a doublet in the recombinant intermediate preparation due to differences in glycosylation (data not shown). The blot was then probed again with anti-GAA-(183–200) antibody (Fig. 3A, lanes 7–9). This revealed the presence of a 10-kDa polypeptide in the intermediate and mature GAA samples (Fig. 3A, lane 8 and 9). These antibodies were helpful to understand the order of the cleavage events as shown in Fig. 3B.

Anti-GAA-(57–74) antibody identified the 110-kDa precursor enzyme, which is consistent with the MS and amino-terminal microsequence analysis of this preparation, but the antibody did not recognize the intermediate preparation, containing the 95/76-kDa forms, or the mature preparation, containing the 76/70-kDa mature forms (Fig. 3B, lanes 1–3). Since amino acids 57–74 were not detected in the intermediate preparation, it can be concluded that the cleavage to release this sequence occurs early in the maturation process. It has not been possible to identify this small peptide by Western blotting using either nitrocellulose or PVDF membranes. It is possible that this small peptide does not bind tightly to the membrane; however, it is more likely lost during processing since the peptide was

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![Summary of the polypeptides and glycans present for each species of GAA](http://www.jbc.org/). Downloaded from http://www.jbc.org/ on September 13, 2017.
not detected by tryptic digestion/MS/MS.

Monoclonal antibody GAA1 (Fig. 3B, lane 4–6) detected the 110-, 95-, 76-, and 70-kDa species, whereas anti-GAA-(183–200) antibody (lanes 7–9). The same blot was then probed with an affinity-purified goat (gt) polyclonal antibody against GAA purified from human placenta (lanes 4–6). The blot was then probed with anti-GAA-(183–200) antibody (lanes 7–9). All blots were developed as described under "Experimental Procedures."

FIG. 3. Identification of the 19.4- and 10.3-kDa polypeptides by Western blotting. The rhGAA precursor (P), rhGAA intermediates (I), and mature human placental GAA (M) were purified as described under "Experimental Procedures." The precursor and intermediates were obtained from the purification of HP-rhGAA. All panels are reducing SDS-polyacrylamide gels (4–20% acrylamide) subjected to Western blotting: A, the GAA precursor, rhGAA intermediates, and mature GAA were probed with anti-GAA-(57–74) antibody (lanes 1–3), monoclonal antibody GAA1 (lanes 4–6), and anti-GAA-(183–200) antibody (lanes 7–9). B, a blot of the GAA precursor, rhGAA intermediates, and mature GAA was probed with monoclonal antibody GAA1 (lanes 1–3). The same blot was then probed with an affinity-purified goat (gt) polyclonal antibody against GAA purified from human placenta (lanes 4–6). The blot was then probed with anti-GAA-(183–200) antibody (lanes 7–9). All blots were developed as described under "Experimental Procedures."

FIG. 4. Identification of a disulfide-bound 3.9-kDa polypeptide. All panels are Western blots of reducing and nonreducing SDS-polyacrylamide gels loaded with the same samples as described in the legend to Fig. 3. A and B are from a 6% acrylamide gel, and C is from a 4–20% acrylamide gel. A, the GAA precursor (P), intermediates (I), and mature GAA (M) were probed with anti-GAA-(57–74) antibody (lanes 1–3) and monoclonal antibody GAA1 (lanes 4–6). B, the blot shown in A was probed with anti-GAA-(78–94) antibody (lanes 1–6). C, the blot from a 4–20% SDS-polyacrylamide gel was probed with anti-GAA-(78–94) antibody. All blots were developed as described under "Experimental Procedures." βME, β-mercaptoethanol.
polypeptide was observed (Fig. 4C, lane 6). The samples were run on a 4–20% SDS-polyacrylamide gel as shown in Fig. 4C. We were not able to detect the 3.9-kDa fragment when run under reducing conditions; however, when the samples were not reduced, an ~14-kDa polypeptide was observed (Fig. 4C, lane 6). The ~14 kDa polypeptide is composed of the 3.9-kDa polypeptide disulfide-bonded to the previously observed 10-kDa polypeptide.

**GAA Exists as a Complex in Other Organisms**—To determine whether cleaved polypeptides are associated with GAA in other tissues, we purified endogenous hamster GAA from CHO cells. To our knowledge, hamster GAA had never before been isolated from a cultured cell line. The purification is described in detail under “Experimental Procedures.” Silver staining/SDS-PAGE analysis of hamster GAA revealed a prominent protein with an apparent molecular mass of 70 kDa (Fig. 4). The 70-kDa protein was isolated by reverse-phase chromatography, and the amino terminus was microsequenced. The amino-terminal sequence determined (APSQLYSVEFSEEPF) is highly homologous to the amino terminus for the human 70-kDa mature enzyme from amino acids 204 to 218 (APSQLYSVEFSEEPF).

The 110-kDa rhGAA precursor, GAA purified from placenta, and hamster GAA purified from CHO cells were compared by SDS-PAGE (Fig. 5A, lanes 1–3). Unlike placental GAA, only the 70-kDa species was present in the highly purified preparation of hamster GAA from CHO cells. Western blot analysis was performed on hamster GAA with anti-GAA-(183–200) antibody (Fig. 5B, lanes 1 and 3) and goat anti-GAA antibody (lanes 2 and 3). The blots show that the 19- and 10-kDa fragments were present in a highly purified preparation of hamster GAA from CHO cells. The presence of these subunits in highly purified GAA preparations from human placenta and CHO cells demonstrates that the formation and association of these subunits are not tissue- or species-specific.

The 19-kDa polypeptide in CHO preparations appears as a doublet. The cDNA for human GAA encodes two glycosylation sites in the 19-kDa fragment. To determine whether carbohydrate differences are responsible for the observed doublet, human placental and hamster GAA were denatured and digested with endoglycosidase H and peptide N-glycosidase F. Peptide N-glycosidase F is an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose-, hybrid-, and complex-type oligosaccharides from N-linked glycoproteins (26). Endoglycosidase H is a glycosidase that cleaves the chitobiase core of high mannose-type and some hybrid-type oligosaccharides from N-linked glycoproteins (26). Endoglycosidase H digestion did not have any affect on the 19.4-kDa polypeptide from human placenta or hamster GAA (Fig. 5C, lanes 1, 2, and 6). This suggests that the carbohydrate is not high mannose-type. However, peptide N-glycosidase F digestion decreased the apparent size of the 19.4-kDa polypeptide for both human placental and hamster GAA (Fig. 5C, lanes 3, 4, 7, and 8). The 19-kDa doublet in the hamster preparation was reduced to a single band, which suggests that glycosylation differences are responsible for the doublet.

**Proteolytic Processing of the 110-kDa Precursor after Uptake in Fibroblasts**—To directly demonstrate that the proteolytic processing of GAA in the cell proceeds via the pathway described, we utilized mannose 6-phosphate receptor-mediated endocytosis of the GAA precursor and a GAA-deficient fibroblast cell line (GM00248) derived from a Pompe’s disease patient. To increase the amount of GAA internalized, HP-rhGAA was used. The protein sequence of HP-rhGAA is identical to that of the rhGAA precursor, but the structure differs in that the N-glycans of HP-rhGAA contain 7–11 mannose 6-phosphate groups/molecule of GAA. This was determined by LC/MS/MS. The evidence for this will be described elsewhere.2 At

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**FIG. 5. Hamster acid α-glucosidase is a multisubunit complex.** rhGAA, human placental GAA, and hamster GAA were purified as described under “Experimental Procedures.” A, silver-stained reducing SDS-polyacrylamide gel (10% acrylamide) of rhGAA (lane 1), human placental GAA (lane 2), and hamster GAA (lane 3). B, Western blot of a reducing SDS-polyacrylamide gel (4–20% acrylamide) of hamster GAA. Lane 1, blot probed with anti-GAA-(183–200) antibody; lane 2, blot probed with affinity-purified goat (gt) anti-GAA antibody raised against GAA purified from human placenta; lane 3, blot probed with antibodies used in lanes 1 and 2. C, Western blot of a reducing SDS-polyacrylamide gel (4–20% acrylamide) of human placental GAA undigested (lanes 1 and 3) and digested with endoglycosidase H (Endo H; lane 2) and peptide N-glycosidase F (PNGase, lane 4) and hamster GAA undigested (lanes 5 and 7) and digested with endoglycosidase H (lane 6) and peptide N-glycosidase F (lane 8) with affinity-purified goat anti-GAA antibody. All blots were developed as described under “Experimental Procedures.”
the designated time points, cell lysates were prepared and analyzed by Western blotting using anti-GAA-(57–74) antibody (Fig. 6A), a monoclonal antibody to the 70-kDa polypeptide (Fig. 6B), and anti-GAA-(183–200) antibody (Fig. 6C). In Fig. 6A, only the GAA precursor standard was identified by anti-GAA antibody. Fragments containing this sequence were not identified in any of the lysate-derived samples. This is consistent with rapid release of this peptide upon internalization. In Fig. 6B, the same blot probed with monoclonal antibody GAA1 shows the conversion of the 110-kDa precursor to the transient 95-kDa intermediate, followed by slower processing to the 76- and 70-kDa forms. In Fig. 6C, a Western blot of the lysates was probed with anti-GAA-(183–200) antibody, demonstrating that the appearance of the 10.3-kDa fragment correlates with the conversion of the 76-kDa form to the 70-kDa form. These results suggest that processing takes place by an ordered and sequential series of steps.

DISCUSSION

It is well known that GAA is synthesized as a 110-kDa precursor of 952 amino acids, yet it exists in lysosomes as major species of 76 and 70 kDa. It is generally agreed that the conversion from the 110-kDa form to the 76- and 70-kDa forms is a result of proteolytic processing at both the amino and carboxyl termini. However, the structures of the 76- and 70-kDa mature forms of the enzyme have not been determined. Furthermore, the details and sequence of the processing steps are incomplete. In this study, we have demonstrated that fully processed GAA consists of four associated polypeptides all derived from the GAA precursor. The ability to determine the structure of each fragment using sensitive mass spectroscopic techniques allows us to propose the model for GAA maturation that is shown in Fig. 7 and supported by Table I.

The GAA predicted primary translation product of 952 amino acids containing seven N-glycans is probably present only transiently and was not detected. The first proteolytic processing step is likely cleavage between amino acids 28 and 29 by signal peptidase; again, the product of this cleavage was not detected. The first precursor identified in this study contains amino acids 57–952, apparently resulting from a proteolytic cleavage between amino acids 56 and 57 (Fig. 7 and Table I). A similar precursor beginning at amino acid 70 has been identified in human urine (10). The first intermediate identified, designated intermediate 1, consists of a 95-kDa polypeptide (amino acids (aa) 122–952) covalently linked via a disulfide bond to a 3.9-kDa polypeptide (aa 78–113). Multiple cleavages are required to generate these fragments. The identification of the 3.9-kDa disulfide-linked polypeptide is entirely novel. Sequences 57–78 and 113–122 are not found, apparently having been lost during processing. The availability of anti-GAA-(57–74) antibody allowed confirmation that this sequence is not present in intermediate 1 (Fig. 3B, lane 2).

Interestingly, almost all of the reduction in molecular mass going from the 110-kDa precursor to intermediate 1 is attributed to the loss of carbohydrate due to extensive glycan trimming. We have not observed glycans on intermediate 1 that were not trimmed. This suggests that the processing that yields intermediate 1 takes place in a late endosome or lysosome, containing multiple glycosidases.

In the next step, the 95-kDa intermediate is proteolytically cleaved at the carboxyl terminus by an unknown protease,
FIG. 7. Model for the maturation of GAA.

Acid $\alpha$-Glucosidase Consists of Four Peptides

FIG. 8. Multiple sequence alignment of human family 31 glucohydrolases. Shown is an alignment of human GAA, maltose isomylase (MGAM), sucrose isomaltase (SIM), glucosidase II (G2AN), and neutral $\alpha$-glucosidase C (GANc). The putative active site (WIDMNE) is highlighted (orange) along with sequences removed during proteolytic processing (green).
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which we have designated as protease 3. The cleaved carboxy-terminal 19.4-kDa fragment (aa 792–952) and the 3.9-kDa fragment remain associated with the 76-kDa polypeptide (aa 122–781). In the final step, the 76-kDa polypeptide is then proteolytically cleaved near the amino terminus by an unknown protease, which we have designated as protease 4, to give the final complex consisting of the following polypeptides: 70 kDa (aa 204–781), 19.4 kDa (aa 792–952), 10.3 kDa (aa 122–200), and 3.9 kDa (aa 78–113). Interestingly, at each cleavage site, four to nine amino acids are not present in the final products (aa 114–121, 200–203, and 783–791); whether this is the result of two specific proteolytic steps or a single cleavage followed by nonspecific aminopeptidase or carboxypeptidase activity has not been determined.

Interestingly, there was no evidence that the three proteolytic processing steps could occur in any order except that proposed in our model. Had these cleavages occurred in a different order, additional intermediates that were not observed would have formed. The understanding that GAA maturation proceeds through a series of discrete ordered steps may also have application as the basis for diagnostic tests for glycogen storage disease type II. In particular, the identification of fragments with masses of 3,927, 10,392, and 19,447 Da might be a sensitive indicator that GAA is present and that maturation has proceeded successfully. Additionally, the detection of such polypeptides could be used to monitor enzyme replacement therapy for Pompe's disease.

The association of the proteolytically cleaved fragments of GAA is not unique for lysosomal enzymes. The lysosomal α-mannosidase enzyme is synthesized as a single chain precursor that is processed into three glycopeptides of 70, 42, and 15 kDa. The 70-kDa glycopeptide is further partially proteolyzed into three more peptides that are joined by disulfide bridges (27).

The previously described GAA2 allele (D91N) (23, 28) is located in the 3.9-kDa fragment (aa 78–113). This substitution results in a decreased affinity of the mature enzyme for starch, leading to the suggestion that a fragment containing the substitution might be associated with the mature enzyme. Our findings present direct physical evidence for both the existence of this fragment and its association with mature GAA. Except for frameshima, disease-causing mutations have been reported in the 10.3-kDa fragment. Several mutations that result in a severe phenotype have been described in the 19.4-kDa fragment (aa 792–952). The in-frame deletion of one amino acid (Lys903) and the common deletion of exon 18 that results in a severe phenotype have been described in the 19.4-kDa fragment and its association with mature GAA. Except for frameshima, disease-causing mutations have been reported in the 10.3-kDa fragment. Several mutations that result in a severe phenotype have been described in the 19.4-kDa fragment (aa 792–952). The in-frame deletion of one amino acid (Lys903) and the common deletion of exon 18 that results in a 55-amino acid deletion from amino acids 828 to 882 both result in the fatal infantile form of the disease (29). Other mutations in the 19.3-kDa fragment include R854X and a frameshift mutation occurring at Pro913, both resulting in severe phenotypes. Prior to this study, it was believed the common exon 18 deletion contained the carboxyl-terminal proteolytic cleavage site based on the belief that the carboxyl-terminal cleavage occurs between amino acids 816 and 881 (3). We have shown that the cleavage site is actually 36 amino acids upstream from the beginning of this common deletion.

The maturation process is not dependent on an active GAA enzyme. A patient with infantile type 2 glycogenosis was found to be homozygous for a E521K substitution (30). When COS cells were transfected with constructs containing the E521K mutation, the GAA was inactive, and GAA maturation was prevented. However, when Glu521 was changed to Gln, maturation of GAA was restored, whereas there was still a loss of activity.

Since mature GAA consists of four polypeptides, this may have implications in treating glycogen storage disease type II. One current strategy for treating the disease is to use enzyme replacement therapy. Using GAA−/− knockout mice (12, 31), several groups have attempted enzyme replacement therapy with precursor and mature forms of recombinant GAA. The 110-kDa rhGAA precursor purified from the milk of transgenic mice and rabbits demonstrated a therapeutic effect in knockout mice (13, 17). Analysis of the tissues revealed that the 110-kDa precursor from rabbit and mouse milk was converted to the 76-kDa lysosomal form. Neither enzyme preparation appeared to be converted significantly to the 70-kDa form. Another study reported using the mature form of GAA (the composition was not described) to reverse muscle weakness in a knockout mouse (15). Our improved understanding of the processing pathway may help us to develop more effective second generation candidates for enzyme replacement therapy.

The sequences of five human family 31 glycosidases (GAA, maltose isoamylase, sucrose isomaltase, glucosidase II, and neutral α-glucosidase C) (16) have been aligned using ClustalW (Fig. 8) (30). Since the maltase isoamylase and sucrose isomaltase sequences have undergone duplication and contain two active sites, they have been divided into the amino- and carboxyl-terminal ends. Aligned in this fashion, the active site (WIDMNE) and many of the cysteines have been conserved. Sequences lost from GAA during processing are indicated in green. It is apparent from the alignment that GAA, maltose isoamylase, and sucrose isomaltase are most closely related, whereas glucosidase II contains a number of insertions and deletions reducing the overall homology. GAA is the only family 31 enzyme that is found in the lysosome and is known to undergo proteolytic maturation/processing. Interestingly, two of the four sequences removed during processing are found in the only two insertions in the GAA sequence relative to the family 31 consensus. It will be interesting in the future to determine how these sequences alter GAA structure and function.

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