Glucosidase II, a Glycoprotein of the Endoplasmic Reticulum Membrane. Proteolytic Cleavage into Enzymatically Active Fragments†

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ABSTRACT: Glucosidase II removes the inner two α-linked glucose residues from freshly transferred Asn-linked oligosaccharide chains in the endoplasmic reticulum. This enzyme, whose activity could be measured by the hydrolysis of an artificial substrate (*p*-nitrophenyl α-D-glucopyranoside), was purified 240-fold from a rat liver microsome fraction by DEAE-cellulose, concanavalin A–Sepharose 4B, and hydroxylapatite chromatography. The apparent molecular weight of the active polypeptide was 123 000 as estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Glucosidase II has at least one high-mannose oligosaccharide chain that can be cleaved by endoglycosidase H. Trypsin readily cleaved the 123-kilodalton (kDa) form of glucosidase II into a fully active 73-kDa core. The pattern of this cleavage suggests a domain structure for this enzyme. We demonstrate that trypsin first removes a glycosylated 25-kDa domain to yield an apparently unglycosylated 98-kDa product which is further cleaved to yield the active 73-kDa core.

A wide variety of oligosaccharide chains are derived from a common precursor (containing three glucose, nine mannose, and two *N*-acetylglucosamine residues) that is transferred from its dolichol pyrophosphate derivative to an asparagine residue of a growing polypeptide in the rough endoplasmic reticulum (Hubbard & Ivatt, 1981). The three glucose residues must be removed before the oligosaccharide can be extensively remodeled in the Golgi apparatus (Grinna & Robbins, 1979). The glucose residues are nitially present as a trisaccharide of the structure $Glc\xrightarrow{\alpha 1,2}Glc\xrightarrow{\alpha 1,3}Glc\xrightarrow{\alpha 1,3}$ attached to a mannose residue. The outer 1,2-linked and inner two 1,3-linked glucose residues are released sequentially by glucosidase I and glucosidase II, respectively, both of which are located in microsomal membranes (Grinna & Robbins, 1979; Burns & Touster, 1982).

A finding that the glucosidase II of rat liver could be specifically assayed with a chromogenic artificial substrate (*p*-nitrophenyl α-D-glucopyranoside) even in crude microsomes greatly facilitated the purification of the enzyme (Burns & Touster, 1982; Michael & Kornfeld, 1980). Here we report a modification of the procedure that results in the purification of a 123-kilodalton (kDa)[1] polypeptide nearly twice the size of that previously obtained (Burns & Touster, 1982). The 123-kDa form of glucosidase II is readily cleaved by trypsin into a 73-kDa fragment that retains full activity and is similar in size to the glucosidase II polypeptide previously described (Burns & Touster, 1982). After this paper was submitted, a report appeared describing the purification of pig kindey microsomal glucosidase II having a subunit molecular weight of about 100 000 (Brada & Dubach, 1984).

Experimental Procedures

Purification of Glucosidase II. Starting material for a typical purification consisted of about 120 g of liver (from 10 rats). All steps were at 4 °C. The activity of glucosidase II was measured by the hydrolysis of *p*-nitrophenyl α-D-glucopyranoside (Burns & Tuster, 1982). The assay mixture contained 50 mM HEPES buffer (pH 6.8), 4 mM substrate, 1% sodium cholate, and an enzyme source in a final volume of 0.5 mL. After incubation for 10–60 min at 37 °C, the reaction was stopped by adding 1 mL of 1% ethylenediamine (pH 10.7), and an absorbance change at 400 nm was measured. The activity was expressed as micromoles of *p*-nitrophenol formed in a given time.

(*1*) *Preparation of Microsomes.* Male Wistar rats, weighing 200–300 g each, were used wihout starvation. Livers were excised, blotted, weighed, and minced in 4 volumes of 0.25 M sucrose solution containing 10 mM Tris-HCl buffer (pH 8.0), 5 mM 2-mercaptoethanol, and a cocktail for inhibition of proteases (1 mM PMSF, 1 mM 1,10-phenanthroline, 1 mM sodium bisulfite, and 2 μg/mL leupeptin). The microsomal fraction was prepared by using 10 mM $CaCl_2$ according to the method reported previously (Burns & Touster, 1982).

(*2*) *Solubilization by a Nonionic Detergent.* Microsomes were suspended in 770 mL (6.5 mL/g of liver) of 50 mM potassium phosphate buffer (pH 7.0) containing 5 mM 2-mercaptoethanol, 2.3 mM EDTA, and the protease inhibitor cocktail described above. Triton X-100 (10%) was added dropwise to the microsomal suspension to make the final detergent concentration 1%. After being stirred for an hour, the mixture was centrifuged at 127000*g* for 90 min to obtain a clear supernatant. Essentially all the glucosidase II activity was extracted into the supernatant fraction.

(*3*) *DEAE-cellulose Column Chromatography.* Binding of enzyme to DEAE-cellulose was achieved in suspension; elution was done from a column. This two-step method greatly improved flow rates and saved time. Swollen DEAE-cellulose (DE-52) was filtered with suction over Whatman 3M paper on a Büchner funnel. Sixty grams of this cake was then added

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[1] Abbreviations: Con A, concanavalin A; Con A–Sepharose 4B, concanavalin A–Sepharose 4B; PMSF, phenylmethanesulfonyl fluoride; TPCK–trypsin, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone–trypsin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Endo-H, endo-β-*N*-acetylglucosaminidase H; NP-40, Nonidet P-40 [octylphenoxypoly(ethoxyethanol)]; PBS, phosphate-buffered saline consisting of 17 mM phosphate buffer (pH 7.3), 0.14 M NaCl, and 2.7 mM KCl; kDa, kilodalton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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to the solubilized supernatant, and the mixture was stirred slowly for about an hour. We found that essentially all the glucosidase II activity bound to the resin. This mixture was then filtered through Whatman 3M paper on a Büchner funnel, and the resulting cake of DEAE-cellulose on the funnel was washed with about 1 L of 50 mM potassium phosphate buffer (pH 7.0) containing 0.1% Triton X-100, 5 mM 2-mercaptoethanol, 1 mM EDTA, and protease inhibitor cocktail. The cake of DEAE-cellulose was then suspended in a minimum amount of the phosphate buffer and formed into a column on top of 100 g of fresh DEAE-cellulose. The resulting column, which had 100 g of fresh DE-52 at the bottom and 60 g of the enzyme-absorbed resin at the top, was washed with 3 column volumes of the same phosphate buffer. The column was eluted with a linear gradient of NaCl (6.5 column volumes, 0–500 mM), at a flow rate of about 100 mL/h. Glucosidase II activity was eluted at about 0.2 M NacL. The peak fractions of the activity were pooled and used for the next step of chromatography.

(*4*) *Con A–Sepharose 4B Column Chromatography.* The pooled fraction from the DEAE-cellulose column was directly applied to a Con A–Sepharose 4B column (1 × 5.5 cm) preequilibrated with 0.1 M potassium phosphate buffer (pH 7.0) containing 0.1% Triton X-100, 5 mM 2-mercaptoethanol, and 0.1 mM each of $CaCl_2$, $MnCl_2$, and $MgCl_2$. The column was washed with 10 column volumes of 0.3 M potassium phosphate buffer (pH 7.0) containing 0.1% Triton X-100, 5 mM 2-mercaptoethanol, 1 mM PMSF, and 2 μg/mL leupeptin and then with the same buffer except that the phosphate concentration was 0.1 M. Glucosidase II was eluted with 0.1 M methyl α-mannoside containing 0.1 M potassium phosphate buffer (pH 7.0), 0.1% Triton X-100, 5 mM 2-mercaptoethanol, 1 mM PMSF, and 2 μg/mL leupeptin (flow rate was about 5 mL/h). The peak fractions of the glucosidase II activity were pooled and dialyzed overnight against 10 mM potassium phosphate buffer (pH 7.0) containing 0.1% Triton X-100, 5 mM 2-mercaptoethanol, 1 mM PMSF, and 2 μg/mL leupeptin.

(*5*) *Hydroxylapatite Column Chromatography.* The dialyzed preparation was applied to a hydroxylapatite column (1.1 × 16.3 cm) preequilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 0.1% Triton X-100 and 5 mM 2-mercaptoethanol. The column was then washed with 3 column volumes of the same buffer (except the phosphate concentration was increased to 20 mM). The column was then eluted volumes in the same buffer. The flow rate was about 5 mL/h. The activity was eluted at about 60 mM phosphate. The peak fractions had two major SDS gel bands (see Figure 4, zero trypsin) and were pooled for use in most of the subsequent experiments.

When the hydroxylapatite column chromatography was repeated, a side fraction which had only one major band (of 123 kDa) was obtained as shown in Figure 3b (the region indicated by the bar was pooled; the SDS gel, see Figure 5, zero trypsin). The concentration of phosphate at which it was eluted was about 40 mM.

(*6*) *Preparative Trypsinization.* For some purposes, the resistance to proteolysis of the glucosidase II activity can be taken advantage of as a purification step. Trypsin–TPCK (0.1 mg/mL) was added to 15 mL of the pooled hydroxylapatite fraction of glucosidase II (final concentration 0.2 μg/mL). After incubation for 16 h at 4 °C, the digest was subjected to the hydroxylapatite column chromatography as described above. This preparation of glucosidase II consisted mainly of the 73-kDa form (see Figure 7a, lanes 2).

Endo-H Digestion of Glucosidase II. The digestion of glucosidase II with Endo-*H* was done as described in Fries & Rothman (1980). The glucosidase II preparation (20 μL, 2–4 μg of protein) was mixed with an equal volume of 0.1 M Tris-HCl buffer (pH 6.8) containing 30 mM dithiothreitol and 2% SDS and heated for 5 min at 100 °C. Then, 20 μL of 0.3 M sodium citrate buffer (pH 5.5) containing 1 mg/mL SDS was added, either containing or lacking Endo-H (6 ng of protein, 2×10^{-4} IU), and incubated for 16 h at 37 °C. The digest was subjected to SDS–PAGE directly or after precipitation with trichloroacetic acid.

Con A Binding. ^{125}I-Labeled Con A was used to determine which proteolytic fragments of glucosidase II retained high-mannose chains. After the untreated and trypsinized preparations of glucosidase II were separated by SDS–PAGE, the polypeptides were transferred to diazonium paper (12 × 16 cm) as described previously (Renart et al., 1979). The paper was thoroughly washed with 0.25% gelatin solution containing 0.1 M Tris-HCl buffer (pH 9.0) and then with a detergent solution [0.15 M NaCl, 50 mM Tris-HCl buffer (pH 7.4), 0.25% gelatin, and 0.05% NP-40] to inactivate the remaining diazonium group. The inactivated paper was incubated for 2 h with 2 mL of the detergent solution containing ^{125}I-labeled Con A (5×10^{-7} cpm; prepared as described below) and then washed extensively with 0.4% *N*-laurylsarcosine containing 50 mM Tris-HCl buffer (pH 7.4) and 0.65 M NaCl to remove unbound ^{125}I-labeled Con A. Kodak X-ray film (XAR-5) was used for the radioautography.

To iodinate Con A, 1 mg of Con A (dissolved in 0.2 mL of PBS containing 0.3 M methyl α-mannoside) was incubated with 0.3 mCi of ^{125}I for 10 min on ice using Iodo-beads (Pierce) as an oxidizing reagent. The reaction was quenched by adding 0.02 mL of 10 mM tyrosine and 0.01 mL of 1 M KI. The ^{125}I-labeled Con A was separated from free ^{125}I by using a Sephadex G-25 column equilibrated with PBS containing 1 mg/mL bovine serum albumin and 0.3 M methyl α-mannoside. The resulting ^{125}I-labeled Con A (1.6×10^5 cpm/μg) was dialyzed exhaustively against PBS to remove methyl α-mannoside.

Other Analytical and Assay Procedures. Protein concentrations were determined by a dye binding method (Read & Northcote, 1981) with bovine serum albumin as a reference. Polyacrylamide gel electrophoresis (7.5% gel concentration) in the presence of SDS and 2-mercaptoethanol was done as described previously (Laemmli & Faure, 1973). Apparent molecular weights were estimated from the mobilities relative to those of standard proteins of known molecular weight: clathrin heavy chain, 180 000 (Pearse, 1975); hexose-6-phosphate dehydrogenase, 108 000 (Hino & Minakami, 1982); bovine serum albumin, 65 000 (Greeth, 1952); egg albumin, 43 500 (Kegeles & Gutter, 1951).

Chemicals and Other Materials. *p*-Nitrophenyl α-(or β)-D-glucopyranoside, Con A–Sepharose 4B, Con A (type IV), and Triton X-100 were purchased from Sigma Chemical Co. DEAE-cellulose (DE52) and hydroxylapatite were obtained from Whatman and Calbiochem, respectively. TPCK–trypsin was obtained from Worthington. ^{125}I was from New England Nuclear. Endo-H was purchased from The Division of Laboratories and Research, New York State Department of Health, Albany, NY. Other chemicals were of reagent grade.

Results

Purification of Glucosidase II from Rat Liver Microsomes. Burns & Touster (1982) have published a purification of glucosidase II in which the product is an essentially homogeneous polypeptide of about 65 kDa. They have shown that,

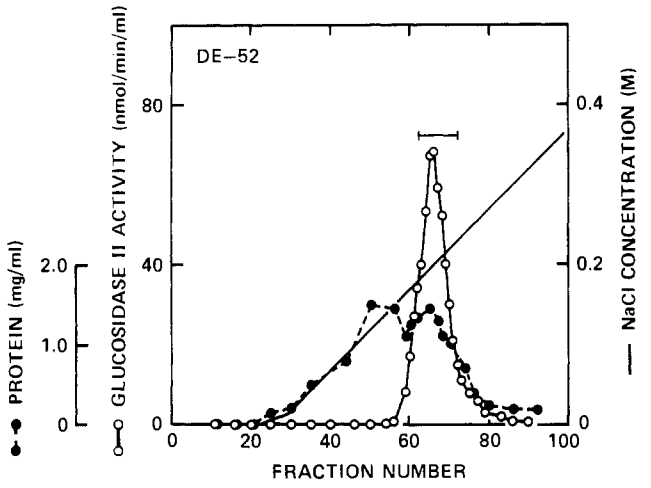

FIGURE 1: DEAE-cellulose column chromatography. After binding of solubilized glucosidase II to DEAE-cellulose in suspension, the column (3.7 × 21.5 cm) was set up, washed, and eluted as described under Experimental Procedures. Fractions of 12 mL were collected, and the elution profiles of glucosidase II activity (○) and protein (●) were determined. A linear gradient of [NaCl] is indicated by the solid line.

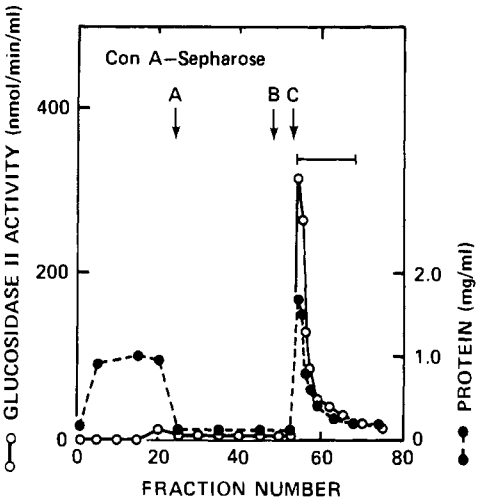

FIGURE 2: Con A-Sepharose 4B column chromatography. The pooled eluate from DEAE-cellulose was applied to Con A-Sepharose 4B (1 × 5.5 cm), and the column was washed (A) with 0.3 M potassium phosphate buffer (pH 7.0) containing Triton X-100, 2-mercaptoethanol, PMSF, and leupeptin and then (B) with the same buffer except that the phosphate concentration was 0.1 M. Glucosidase II was eluted with 0.1 M methyl α-mannoside (C), and the activity (○) and protein (●) distributions were determined. Fractions of 7 mL (fractions 1–54) or 2.8 mL (fractions 54–80) were collected.

when washed rat liver microsomal fraction is used as a starting material for purification, the *p*-nitrophenyl α-D-glucopyranosidase activity is copurified with the activity to cleave glucose residues from $Glc_{1-2}Man_9GlcNAc_2$ (so-called glucosidase II). Michael & Kornfeld (1980) have also reported the same observation using calf liver microsomes, and Elting & Lennarz (1982) reported that purified hen oviduct glucosidase I did not show any measurable activity toward this artificial substrate, strongly indicating that glucosidase II activity can be assayed as a *p*-nitrophenyl α-D-glucopyranosidases activity during the purification of the enzyme. In the purification of Burns & Touster (1982), detergent was used to extract the enzyme, but was not employed at most of the later steps. They also found that the enzyme was highly unstable at several stages in its purification. It is perhaps because of the instability of the enzyme that we encountered difficulty in obtaining pure enzyme by following their procedure. We therefore undertook to modify the published procedure by including Triton X-100 throughout the purification.

Briefly, after solubilization from microsomes, glucosidase II was purified by chromatography on DEAE-cellulose, concanavalin A–Sepharose 4B, and hydroxylapatite, all in the presence of Triton X-100. A single major peak of activity eluted from the DEAE column (Figure 1) and was applied directly to the Con A–Sepharose 4B column (Figure 2). Finally, the eluate from the Con A–Sepharose 4B column was applied to a hydroxylapatite column (Figure 3a). A typical purification is summarized in Table I. About 7 mg of protein could be obtained from about 120 g of rat liver with a yield of 33% (first hydroxylapatite column, Figure 3a). The purified prepartaion of glucosidase II did not show any detectable activity toward *p*-nitrophenyl β-D-glucospyranoside. The enzyme was stable for many days at 4 °C in its purest form as well as at all intermediate steps.

The pool from the hydroxylapatite column consisted of two major polypeptides of molecular weights 123 000 and 90 000 (see Figure 4, lane to the left marked as 0 trypsin). Although the glucosidase II activity was purified to a degree comparable to that reported by Burns and Touster (using the same assay and source of enzyme), we could detect no 65-kDa polypeptide in our preparation; yet this was the major product of theirs. We therefore suspected that their 65-kDa form of glucosidase

Table I: Summary of Purification of Glucosidase II from Rat Liver Microsomes

steps	protein (mg)	volume (mL)	glucosidase II activity: units[a]	glucosidase II activity: units/mg of protein
(1) microsomes	5456	770	9.94 (100)[b]	0.0018 (1.0)[c]
(2) solubilized supernatant	3374	865	9.96 (100)	0.0030 (1.6)
(3) DEAE-cellulose	176	120	5.67 (57)	0.0323 (17.7)
(4) Con A-Sepharose 4B	25.4	48	3.71 (37.3)	0.146 (80.3)
(5) first hydroxylapatite[d]	7.52	40	3.32 (33.4)	0.442 (243)
(6) limited trypsinization	2.66		2.08 (20.9)	0.782 (430)

[a] One unit was defined as 1 μmol of *p*-nitrophenol formed/min. [b] Percent recovery of activity in parentheses. [c] *x*-fold purification in parentheses. [d] The result of the first hydroxylapatite column chromatography (Figure 3a) was presented. This was the standard preparation used for most parts of this experiment.

II was a proteolytic degradation product of a more intact form of the enzyme, possibly the 123-kDa or the 90-kDa polypeptide in our preparation.

When the pooled fraction eluted from hydroxylapatite was rechromatographed on hydroxylapatite, a highly purified preparation of glucosidase II could be thus obtained as a side fraction, albeit in very small amounts (Figure 3b, pool indicated by the horizontal bar). We found that the 123-kDa component coeluted with activity from the hydroxylapatite (Figure 3b, insert). We could not accurately determine the specific activity of this side fraction because the protein concentration was too low (<0.02 mg/mL) to yield a reliable value. This enzyme of the side fraction consisted almost entirely of the 123-kDa component (Figure 3b, insert, fractions 18–20), with little or none of the previously found 90-kDa component. The trace contaminant can be accounted for as proteolysis products of the 123-kDa and (see below). Therefore, it seemed likely that the 123-kDa band rather than the 90-kDa component in our standard glucosidase II prepa-

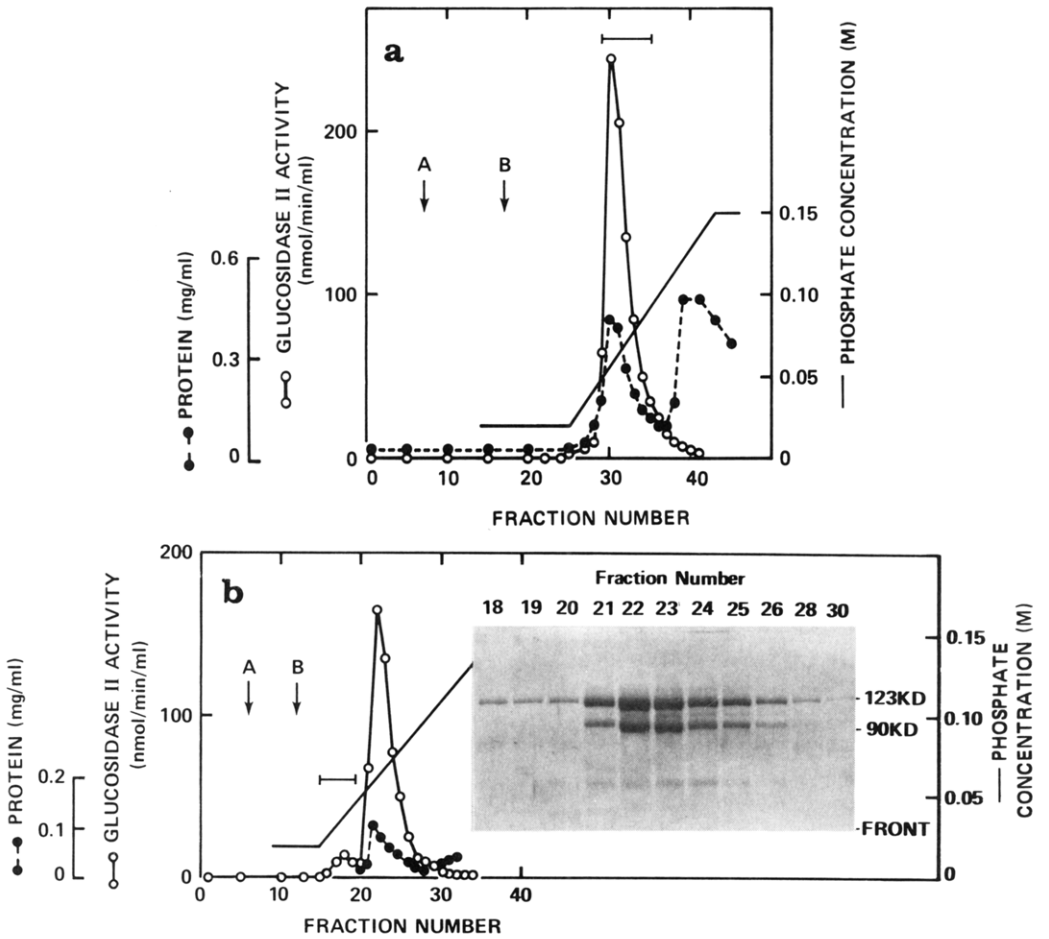

FIGURE 3: Hydroxylapatite column chromatography. (a) The pooled eluate of glucosidase II from the Con A–Sepharose 4B column was applied to hydroxylapatite column (1.1 × 16.3 cm) and washed (A and B), as described under Experimental Procedures. Glucosidase II was eluated by increasing the concentration of phosphate buffer (—), and the elution profiles of glucosidase II activity (○) and protein (●) were determined. Fractions of 7.7 mL (fractions 1–16) or 4.2 mL (fractions 17–45) were collected. (b) The pool of glucosidase II activity from hydroxylapatite column chromatography in (a) (indicated by the horizontal bar) was subjected to the rechromatography of hydroxylapatite (1.3 × 11 cm). After the column was washed as before (A and B), glucosidase II was eluted by increasing the concentration of phosphate buffer as above. Fractions of 4.5 mL were collected. A pool of side fractions (horizontal bar) was made that was especially enriched in the 123-kDa component. The insert shows SDS–PAGE patterns of both side and major fractions of activity, demonstrating that the 123-kDa component is responsible for the activity. Protein concentrations in the side fractions were too low (<0.02 mg/mL) to be accurately measured.

ration (Figure 4) represented the actual enzyme. The 65-kDa form purified earlier (Burns & Touster, 1982) would represent a product of proteolysis during purification.

Trypsinization of Glucosidase II. To test the idea that the previously reported 65-kDa form of glucosidase II was a proteolysis product of the 123-kDa enzyme, we purposefully added trypsin to our enzyme to see if it could be degraded to a catalytically active fragment of similar size.

Figure 4 shows the effect of increasing amounts of trypsin upon the activity of a standard preparation of glucosidase II and upon its SDS–PAGE pattern. The activity is almost entirely resistant to a 5-min digestion at 37 °C up to a weight ratio of about 0.08 μg of trypsin per μg of protein. The SDS gel reveales that the 123-kDa component has been quantitatively digested, and only a 73-kDa fragment remains. All detectable impurities have also been digested. Indeed, when this is done preparatively, a further enrichment of activity occurs (Table I, step 6). The 90-kDa component is removed well before the 123-kDa component is cleaved (Figure 4, 0.002–0.01 trypsin), showing that 90 kDa is not glucosidase II and that the 73-kDa limit digestion product must derive from the 123-kDa band.

The conclusion that the 123-kDa polypeptide is cleaved into a stable 73-kDa and active fragment was confirmed by tryptic dissection of the more purified fraction of glucosidase II obtained by repeating the hydroxylapatite step (Figure 5). Here it is clear that the 73-kDa band must derive from the 123-kDa component without loss of activity. Altogether, these experiments strongly suggest that glucosidase II activity resides in a 123-kDa polypeptide. This protein can be cleaved by trypsin into a catalytically active core of about 73 kDa that retains full activity.

Domain Structure of Glucosidase II. The ability to cleave off a catalytically active core suggests that this glucosidase has a domain structure. Frequently, the boundaries that connect independently folded domains in the proteins are the sites at which proteases first attack. In the digestion of the 123-kDa form of glucosidase II, we noted the transient but quantitatively significant accumulation of a 98-kDa intermediate (Figures 4 and 5). This would suggest that the first cleavage by trypsin removed a domain of about 123 − 98 = 25 kDa, resulting in the observed 98-kDa active fragment, which is itself relatively resistant to further trypsin attack. The next cleavage(s) result(s) in the 3-kDa active core as a limit digestion product (at least under these conditions). However, a longer incubation at lower temperature (16 h at 4 °C) and lower trypsin/sample ratio resulted in the digestion of the core with resulting loss of activity (Figure 6). No other stable or active digestion products could be detected; rather, it appears that further digestion of the 73-kDa core at any of a number

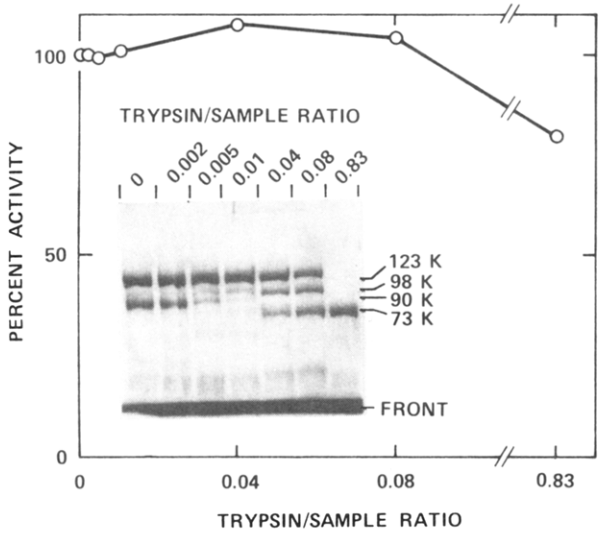

FIGURE 4: Stepwise degradation of glucosidase II with trypsin. Samples of the glucosidase II preparation (2.5 μg of protein each) obtained from the hydroxylapatite column (step 5) were incubated for 5 min at 37 °C in a reaction mixture (0.2 mL) consisting of 30 mM potassium phosphate buffer (pH 7.0), 0.05% Triton X-100, 5 mM 2-mercaptoethanol, and the indicated amount of TPCK–trypsin [Xpressed as ratio of trypsin concentration (in micrograms) to sample protein concentration (in micrograms)]. The reaction was stopped by adding 10 μg of soybean trypsin inhibitor and cooled on ice. Half of each incubation was subjected to SDS–PAGE, and the other half was assayed for glucosidase II activity (expressed as the percent of the initial activity). The broad band at the bottom of each lane is the soybean trypsin inhibitor.

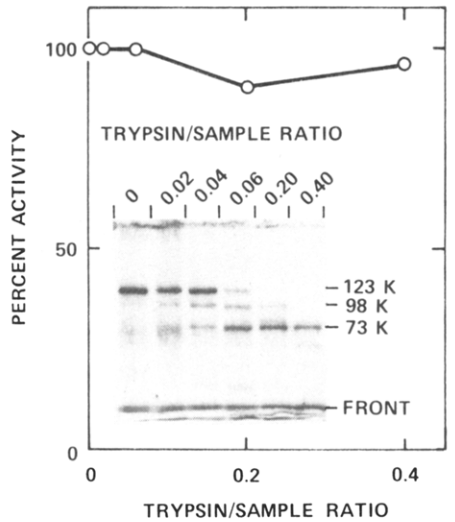

FIGURE 5: Stepwise degradation of glucosidase II with trypsin. The side fractions of glucosidase II obtained with a second hydroxylapatite column (horizontal bar in Figure 3b) was incubated with trypsin in the same manner as in Figure 4 except that leupeptin was added in place of soybean trypsin inhibitor to stop the reaction.

of sites must result in a concerted loss of activity.

The native 123-kDa glucosidase is presumably a glycoprotein with mannose-containing oligosaccharides because it binds to and is eluted from Con A–Sepharose during its purification. Which of its domains are glycosylated, and what sort of oligosaccharide structures are present? Figure 7a shows that the 123-kDa enzyme is Endo-H sensitive but that the 98-kDa digestion intermediate and the 73-kDa core domains are not. This shows that at least one high-mannose-type Asn-linked oligosaccharide chain is attached to the native 123-kDa glycoprotein and that the glycosylated domain is removed during the cleavage of the 123-kDa component to

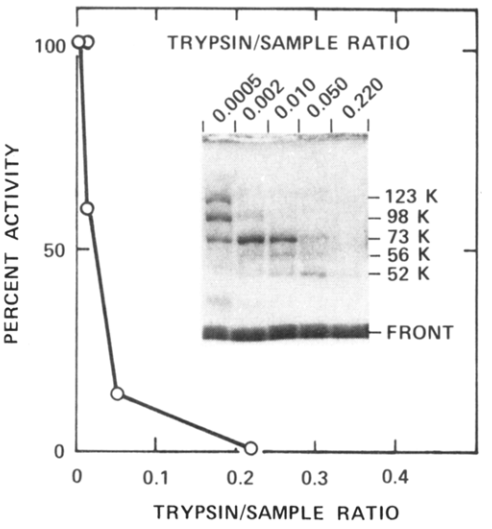

FIGURE 6: Stepwise degradation of glucosidase II with trypsin. Same as in Figure 5 except that the incubation was conducted for 16 h at 4 °C.

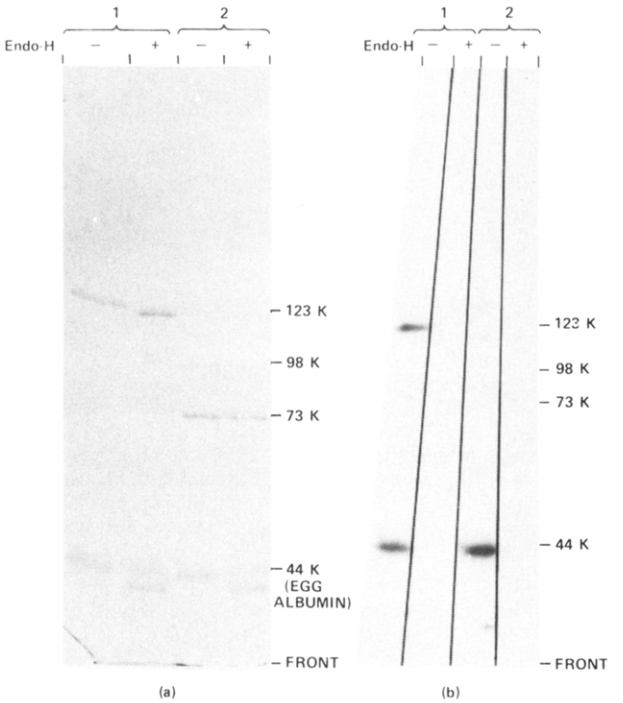

FIGURE 7: Effects of Endo-H treatment on glucosidase II. The untreated (group 1, step 5 under Experimental Procedures) and trypsinized (group 2, step 6 under experimental Procedures) preparations of glucosidase II were incubated with (+) or without (–) Endo-H and then subjected to SDS–PAGE in the presence of 2-mercaptoethanol. Half of the gel was stained for protein with Coomasie Brilliant Blue R250 (a). A duplicate half was analyzed for ^{125}I-labeled Con A binding (b). The intact form of glucosidase II (123 kDa) was the only polypeptide sensitive to Endo-H digestion and able to bind ^{125}I-labeled Con A. Egg albumin (2 μg) was included in the samples as an internal standard.

yield the 98-kDa intermediate. Figure 7b shows a Western-type blot of an SDS–PAGE gel of a tryptic digestion in which a paper onto which peptides were transferred from the gel was incubated with ^{125}I-labeled Con A to reveal polypeptides containing Asn-linked oligosaccharides. As expected, the 123-kDa band but not the 98- or the 73-kDa band binds Con A. All of the ability of the 123-kDa glycoprotein to bind Con A is eliminated by Endo-H digestion. These data confirm that the glycopeptide(s) is (are) removed during the 123-kDa → 98-kDa cleavage. These data do not exclude the possibility,

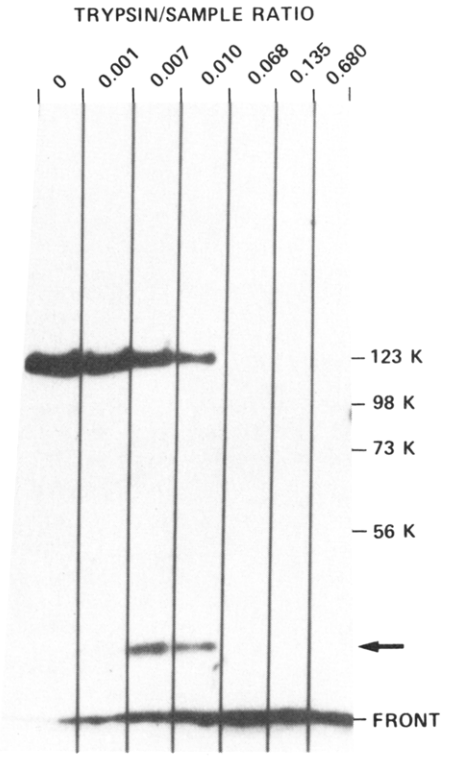

FIGURE 8: Kinetics of trypsin digestion of glucosidase II studied by ^{125}I-labeled Con A binding. Glucosidase II obtained from repeat hydroxylapatite column chromatography (Figure 3b) was incubated for 5 min at 37 °C with increasing concentrations of TPCK–trypsin at the indicated ratios of trypsin concentration (in micrograms) to sample protein concentration (in micrograms). A blot of the SDS gel of these samples was stained with ^{125}I-labeled Con A, as described under Experimental Procedures. The arrow indicates the 25-kDa glycosylated fragment.

however, that Asn-linked complex-type oligosaccharides that are resistant to Endo-H are present on the glucosidase II molecule. These oligosaccharides, if localized either in the 98-kDa intermediate or in the 73-kDa core domain, might not bind Con A under the conditions employed in the Western-type blotting.

These results all point to the idea that the first step in tryptic cleavage is the removal of a 25-kDa glycosylated domain. Presumably this domain would itself be cleaved further, since stoichiometric amounts of a 25-kDa glycoprotein do not accumulate (Figures 4 and 5). The high sensitivity of the radiolabeled Con A blotting method (Figure 7b) encouraged us to look for this glycosylated fragment at intermediate stages of digestion using this technique. Indeed, we found the transient appearance of a species that binds Con A (Figure 8, arrow). This glycopeptide turns out to be of apparent molecular weight 25 000, appears and disappears at the appropriate stages of the digestion (compare Figure 8 with Figure 4), and clearly can only be derived from the major 123-kDa band in Figure 8.

Discussion

We have extensively purified glucosidase II and offered evidence to suggest that its activity resides in a 123-kDa glycoprotein. We could not detect the 65-kDa protein purified by Burns & Touster (1982) in our preparations, even though ours were comparably enriched to theirs using the same assay to follow purification. The simplest explanation is that we have have purified a more intact version of the enzyme. In support of this, we demonstrated that our enzyme could be converted by controlled proteolysis into a fully active core of 73 kDa that was very resistant to further attack by trypsin. When further hydrolysis does eventually occur, activity is lost in a concerted fashion. The major differences between our purification and theirs are (1) that we included a cocktail (consisting of PMSF, phenanthroline, bisulfite, and leupeptin) whose intent was to inhibit endogenous proteolysis and (2) we retained detergent during the purification. This cocktail has been very helpful in other circumstances to minimize proteolysis during purifications (Villani et al., 1980). Brada & Dubach (1984) have independently reported a similar observation. They have isolated an essentially homogeneous glucosidase II preparation from pig kidney microsomes and found that the subunit molecular weight of the enzyme was about 100 000, evidently larger than that reported by Burns & Touster (1982).

The 73-kDa tryptic core reported here is likely to be related to but not identical with the 65-kDa form whose purification was described earlier (Burns & Touster, 1982). Our core lacks the ability to bind Con A, whereas the 65-kDa protein obtained by previous investigators (Burns & Touster, 1982) is apparently a glycoprotein, judging from its binding to a Con A–Sepharose column as a part of the purification procedure. Evidently, when trypsin releases the active "core" domain, it clips off the oligosaccharide(s). When the unidentified endogenous protease(s) release(s) the active 65-kDa form of the core, the oligosaccharide is retained, cleavage being at nearby but different sites. Indeed, it is a common experience that different proteases release the same domain by cleaving at any of several nearby sites at an exposed interface between domains. This apparent discrepancy is thus easily resolved and even suggests that the oligosaccharide(s) of the core are located at the extreme N- or C-terminal side of the segment of the polypeptide chain that gives rise to this domain.

Registry No. Glucosidase II, 9073-99-8.

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