Purification and Multimeric Structure of Bovine N-Acetylglucosamine-1-phosphodiester α-N-Acetylglucosaminidase*

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N-Acetylglucosamine-1-phosphodiester α-N-Acetylglucosaminidase (EC 3.1.4.45; phosphodiester α-GlcNAcase) catalyzes the second step in the synthesis of the mannose 6-phosphate determinant required for efficient intracellular targeting of newly synthesized lysosomal hydrolases to the lysosome. A partially purified enzyme from bovine pancreas was used to generate a panel of murine monoclonal antibodies. The anti-phosphodiester antibody was used to immunopurify the bovine liver enzyme and used to identify the bovine liver enzyme activity. The subunit structure of the enzyme was determined using a combination of analytical gel filtration chromatography, SDS-polyacrylamide gel electrophoresis, and amino-terminal sequencing. The data indicate that bovine phosphodiester α-GlcNAcase is a 272,000-Da complex of four identical 68,000-Da glycoprotein subunits arranged as two disulfide-linked homodimers. A soluble form of the enzyme, isolated from fetal bovine serum, showed the same subunit structure. Both forms of the enzyme reacted with a rabbit antibody raised to the amino-terminal peptide of the liver enzyme, suggesting that phosphodiester α-GlcNAcase is a type I membrane-spanning glycoprotein with its amino terminus in the lumen of the Golgi apparatus.

EXPERIMENTAL PROCEDURES

Materials

Bovine pancreas was obtained from a local slaughterhouse, and bovine calf livers were obtained from Pel Freeze Inc. (Rogers, AR). Superoxide 6, prep grade, Superoxide 6HR 10/30 columns, QAE-Sepharose A-25, concanavalin A-Sepharose, Blue Sepharose CL-6B, and Sephrose Fast Flow, and molecular mass standards for SDS-PAGE were obtained from Amersham Pharmacia Biotech. UltraLink™ BioSupport Medium ABI, UltraLink™ immobilized protein A-Plus, and the BCA protein assay reagent were obtained from Pierce. DE-52 DEAE-cellulose was from Whatman (Haverhill, MA). PNGase F and Endo Hf were from New England Biolabs (Beverly, MA). ProBlott membranes were from Applied Biosystems (Santa Barbara, CA), and POROS 50 HQ was from PerSeptive Biosystems (Cambridge, MA). Iscove’s medium was from Fisher (St. Louis, MO). LB broth was from ICN (Costa Mesa, CA). Centriprep and Centricron concentrators were from Amicon (Beverly, MA). [3H]UDP-GlcNAc was from ARC (St. Louis, MO). Precast polyacrylamide gels and buffers were from Novex (San Diego, CA). ECL reagents were from Amersham Pharmacia Biotech.

α-GlcNAcase, N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase; MOPS, 3-(N-morpholino)propanesulfonic acid; mAb, monoclonal antibody; Endo Hf, endoglycosidase Hf; PNGase F, peptide: N-glycosidase F.

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Methods

Protein Determination

Protein concentration was estimated by absorbance at 280 nm assuming $E_{1\text{cm}}^{1%} = 10.0$ or using the Micro BCA assay standardized with bovine serum albumin (8).

Sialic Acid Determination

The sialic acid content of the bovine liver phosphodiester α-GlcNAcase was determined following mild acid hydrolysis (2 M acetic acid, 80 °C, 3 h). The released sialic acid was derivatized with the fluorescent tag 1,2-diamino-4,5-methylenedioxybenzene and quantitated using reverse-phase high pressure liquid chromatography with online fluorescence detection.

SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out in MOPS buffer on 10% or 4–12% gradient polyacrylamide gels. Proteins were visualized by silver staining (9).

Glycosidase Digestion of Phosphodiester α-GlcNACase

Aliquots (2 μg) of phosphodiester α-GlcNAcase were denatured in 0.5% SDS, 1% β-mercaptoethanol at 100 °C for 10 min. PNGase F (1 milliunit) or Endo Hf was added, and the incubation was continued overnight at 37 °C. As a control, boiled enzyme was added to parallel incubations. The samples were then subjected to SDS-PAGE, and protein bands were detected by silver staining.

Amino-terminal Protein Sequencing of the Bovine Liver Phosphodiester α-GlcNAcase and Generation of Rabbit Polyclonal Anti-peptide Antibodies

Phosphodiester α-GlcNAcase (20 μg) was absorbed onto a ProBlott membrane and subjected to automated Edman degradation in an Applied Biosystems model 478 sequencer. The amino acid sequence of the amino-terminal 33 amino acids was as follows: Asp-Xaa-Thr-Arg-Val-His-Ala-Gly-Ala-Arg-Leu-Glu-Glu-Ser-Trp-Pro-Pro-Ala-Ala-Gln-Thr- Ala-Gly-Ala-His-Arg-Pro-Ser-Val-Ara-Thr-Phe-Val. A 14-amino acid peptide corresponding to NH$_2$-Thr$_3$-Trp$_5$-Cys-COOH was synthesized and purified by BioSynthesis Inc. (Lewisville, TX). The peptide (2 mg) was coupled to 7 mg of keyhole limpet hemocyanin from Puce using 10 mM glutaraldehyde in a 1.0-mL reaction mixture that was incubated overnight with end-over-end rotation at room temperature. The peptide/keyhole limpet hemocyanin conjugate was stored at 4 °C until 0.5 ml was used to immunize a rabbit after being emulsified with 0.7 ml of complete Freund’s adjuvant (Life Technologies, Inc.). The rabbit was boosted 1 month later using 0.5 ml of peptide/keyhole limpet hemocyanin conjugate emulsified in 0.7 ml of incomplete Freund’s adjuvant (Life Technologies, Inc.). The rabbit was bled at 2-week intervals after the boost, and these sera as well as preimmune serum from the rabbit were tested for anti-peptide antibody titer by dot blots of the peptide developed with horse-radish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Pharmacia Biotech) and detected with ECL (Amersham Pharmacia Biotech). The anti-peptide antibody was purified from the serum by adsorption to a 2.0-ml affinity column consisting of 4 mg of peptide antigen coupled to 15 mM Imidazole in 25 mM Tris, 150 mM NaCl, 0.2% Lubrol and developed at a flow rate of 1.0 ml/min. The enzyme activity eluted in a single peak at the leading edge of the major protein peak. The fractions containing activity were combined and dialyzed against 5 mM Tris-maleate, pH 7.4, 0.2% Lubrol.

Generation and Identification of Monoclonal Antibodies Specific for Phosphodiester α-GlcNAcase

Buffers

Buffer A contained 0.010 mM Tris-maleate, pH 6.7, 1% Triton X-100, 0.15 mM NaCl, 1 mg/ml bovine serum albumin. Buffer B contained 0.010 mM Tris-maleate, pH 6.7, 1% Triton X-100, 1 mg/ml bovine serum albumin. Buffer C is 0.150 mM Tris-maleate, pH 6.7, 1.5% Triton X-100. Buffer D is 0.05 mM Imidazole-HCl, pH 7.0, 0.15 mM NaCl, 0.01 mM EDTA. Buffer E is 0.025 mM Tris-HCl, pH 7.4, 0.3% Lubrol. Buffer F is 0.5 mM NaHCO$_3$, pH 8.0, 0.3% Lubrol. Buffer G is 0.5 mM NaHCO$_3$, pH 10.0, 0.3% Lubrol.

Polyclonal antisera that immunocapture phosphodiester α-GlcNAcase were raised in BALB/c mice inoculated with 5 μg of partially purified bovine pancreatic enzyme (described above) (specific activity 6000 units/mg). Monoclonal antibodies were prepared by fusing spleen cells from an immunized mouse to SP2/0 mouse myeloma cells according to our modification (2) of standard procedures (11). The fusion was plated in eight 96-well plates and grown in Iscove’s medium containing 20% fetal bovine serum and 10 ng/ml recombinant human interleukin-6 (12) until hybridomas were visible in most wells.

Hybridomas secreting phosphodiester α-GlcNAcase-specific monoclonal antibodies were identified through a three-stage screening procedure described below and summarized in Table II. In the first step, primary pools were constructed from aliquots (50 μl) of supernatants from 16 wells and screened for their ability to capture phosphodiester α-GlcNAcase activity. Enzyme (1.2 units) in 200 μl of buffer A was incubated with pooled hybridoma supernatant (800 μl) overnight at 4 °C. UltraLink™ Immobilized Protein A Plus coated with rabbit antiserum IgG (30 μl of a 1:1 slurry in buffer A) was added, and the incubation
Immunoaffinity Purification of Phosphodiester α-GlcNAcase

Preparation of [3H]GlcNAc-labeled Uteroferrin

Uteroferrin (4 nmol) was incubated with 3000 units of bovine mammary gland GlcNAc-phosphotransferase (2), 30 μmol of [3H]UDP-GlcNAc (specific activity 57 Ci/mmol), for 16 h at 37 °C in the presence of 20 μl Tris-HCl, pH 7.5, 10 mM GlcNAc, 1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 1.2 mM ATP, 5 mM MgCl₂, and 5 mM MnCl₂. The reaction mixture was then chromatographed on a PD-10 column packed with Sephadex G-25 equilibrated with 50 mM Tris-HCl, pH 7.5, and the [3H]GlcNAc-uteroferrin was isolated from the void fraction.

RESULTS

Generation and Identification of Monoclonal Antibodies Specific for Phosphodiester α-GlcNAcase—Phosphodiester α-GlcNAcase used as antigen to prepare monoclonal antibodies was partially purified about 10,000-fold as described under “Experimental Procedures” and summarized in Table I. BALB/c mice were immunized, spleens removed, fused with SP2/0 myeloma cells as described under “Experimental Procedures,” and plated in eight 96-well plates. Following selection, hybridomas secreting anti-phosphodiester α-GlcNAcase were identified by screening pools of hybridoma supernatants. Screening the initial pools of 16 wells identified four pools able to capture phosphodiester α-GlcNAcase activity above background. After three rounds of screening, three of these four pools were found to contain a single hybridoma secreting anti-phosphodiester α-GlcNAcase (Table II). Hybridomas UC2 and UC3 secreted low affinity monoclonals, while hybridoma UC1 was found to secrete a high affinity monoclonal antibody. mAb UC1 was prepared in ascites culture and investigated for use in immunoaffinity isolation of phosphodiester α-GlcNAcase.

Properties of Monoclonal Antibody UC1—The mouse monoclonal antibody UC1 secreted into tissue culture supernatant was typed as an IgG1 subclass using the Sigma immunotype mouse monoclonal antibody isotyping kit. The UC1 mAb was quite effective in the enzyme capture assay, but when we used increasing amounts of antibody to titrate its ability to immunoprecipitate a constant amount of phosphodiester α-GlcNAcase, we found less and less enzyme activity in the pellets. This anomalous result suggested that mAb UC1 inhibited enzyme activity, and this possibility was tested directly as shown in Fig. 1. Both mAb UC1 and an irrelevant mouse monoclonal antibody were precipitated from tissue culture supernatants with 50% saturated (NH₄)₂SO₄, and the pellets were redissolved in and dialyzed against Tris-buffered saline to concentrate the antibodies, which were then adjusted to the same concentration before use. As shown in Fig. 1, increasing amounts of mAb UC1 caused a progressive and hyperbolic inhibition of the activity of a fixed amount of partially purified bovine liver phosphodiester α-GlcNAcase, whereas increasing amounts of the irrelevant mAb did not significantly inhibit the enzyme activity. This result supports the conclusion that mAb UC1 binds directly to phosphodiester α-GlcNAcase. As described under “Experimental Procedures,” the mAb UC1 when

Immunoaffinity Purification of Phosphodiester α-GlcNAcase from Bovine Liver

Step 1: Homogenization, Isolation of Membranes, and Solubilization—Frozen bovine calf liver (1 kg) was thawed at 4 °C and homogenized for 2 min in a Waring blender (high speed) in 2.4 liters of buffer D at 25 °C. Membranes were then collected by centrifugation at 30,000 × g for 30 min; the membrane pellet was rehomogenized in 2.4 liters of buffer D two or three times. Membrane proteins were solubilized in 750 ml of buffer D containing 2% Triton X-100, 0.5% deoxycholate, and insoluble material was removed by centrifugation before, generating a solubilized membrane fraction. The solubilized membrane fraction was combined with 20 ml of UC1-UltraLink™ and continuously rotated at 44 rpm on a rotary tumbler for 16 h at 4 °C. In some cases, the solubilized membrane proteins were incubated with phosphomannan-UltraLink™ to isolate the cation-independent mannose 6-phosphate/insulin like growth factor II receptor before adsorption of the phosphodiester α-GlcNAcase.

Step 2: UC1-UltraLink™ Chromatography—The UC1-UltraLink™ was collected by centrifugation (2000 × g; 10 min) in 250-ml conical bottles. The UC1-UltraLink™ was packed into a 2.6 × 20-cm column and washed with buffer E at 5 ml/min until the absorbance of the eluate at 280 nm was less than 0.05. Non-specifically bound proteins were eluted next by washing with 2 column volumes of buffer F. Phosphodiester α-GlcNAcase was then eluted with buffer G. Fractions of 1 ml were collected in 100 ml of 1.0 M Tris-HCl, pH 5.5, to reduce the pH. Fractions were assayed for protein (absorbance 280 nm) and phosphodiester α-GlcNAcase activity, and fractions containing significant enzyme activity were pooled.

Step 3: Superoxere 6 Chromatography—Pooled fractions from UC1-UltraLink™ were diluted 20-fold with 0.3% Lubrol and applied to a 0.5 × 1.0-cm column of POROS-50 HQ equilibrated with buffer E at a flow rate of 2.0 ml/min. Phosphodiester α-GlcNAcase was step-eluted with buffer E containing 0.5 μM NaCl. Fractions containing the highest absorbance were further concentrated in a Centricon 100 concentrator to 0.5 μl. The concentrated enzyme was applied to a Superose 6 HR 10/30 column equilibrated with buffer E containing 0.15 μM NaCl. The column was developed at a flow rate of 0.25 ml/min; 0.25-ml fractions were collected and assayed for protein (absorbance 280 nm) and phosphodiester α-GlcNAcase activity. Protein was assayed in pooled fractions by a BCA assay.

Immunoaffinity Purification of Phosphodiester α-GlcNAcase from Fetal Calf Serum

Essentially the same procedure was used to purify the soluble form of phosphodiester α-GlcNAcase from 2.5 liters of outdated fetal calf serum (Harlan Bioproducts, Indianapolis, IN), except Lubrol was left out of all of the buffers, since the serum enzyme is soluble. After a preliminary precipitation with 50% saturated ammonium sulfate and thorough dialysis against buffer E without Lubrol, the enzyme was adsorbed to 1.5 ml of UC1-UltraLink™ and washed and eluted as above. The pooled fractions from the UC1-UltraLink™ were dialyzed to remove the pH 10 bicarbonate and concentrated in a Centricon before gel filtration on the Superoxere 6 HR 10/30 column.

TABLE I
Partial purification of phosphodiester α-GlcNAcase from bovine pancreas to use as antigen

| Fractionation step | Specific activity nmol/h/mg |
|--------------------|---------------------------|
| 1. Triton X-100-solubilized membranes | 0.6 |
| 2. Concanavalin A-Sepharose eluate | 36 |
| 3. Blue Sepharose run-through | 65 |
| 4. Cu²⁺-chelating Sepharose eluate | 225 |
| 5. Centriprep-50 concentration of step 4 fractions | 428 |
| 6. Superose 6 gel filtration | 6,924 |
| 7. DEAE-cellulose eluate | 6,061 |

Immunoaffinity Purification of Phosphodiester α-GlcNAcase

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continued for 1 h with mixing. The beads were then washed four times with 1 ml of buffer A, followed by four washes with buffer B. After each wash, the Protein A-UltraLink™ was collected by centrifugation in a microcentrifuge for 5 min, and the supernatant was aspirated. The washed protein A-UltraLink™ pellets were assayed for phosphodiester α-GlcNAcase activity following the addition of 10 μl of buffer C containing 1.5 mM [3H]GlcNAc-phosphomannose-α-methyl and incubation at 37 °C for 4 h. The reaction was stopped by the addition of 1 ml of 2 mM Tris base, and the reaction mixture was chromatographed as described previously (10). One day later, secondary pools of four wells were constructed from aliquots (200 μl) of the wells composing the positive primary pools. These 16 secondary pools were assayed as before. One day later, aliquots of media (200 μl) from single wells comprising the positive secondary pools were assayed. Using this capture assay, hybridomas secreting specific monoclonal antibodies against phosphodiester α-GlcNAcase were subcloned twice, and antibody was produced in ascites culture (11).
coupled to UltraLink™ Biosupport medium, proved to be a very specific and efficient affinity adsorbent for phosphodiester α-GlcNAcase. In data to be presented later, the UC1 antibody was shown to react in Western blots only with the enzymatically active dimer form of bovine phosphodiester α-GlcNAcase. Immunoaffinity Purification of Phosphodiester α-GlcNAcase—mAb UC1 was immobilized on UltraLink™ and formed the basis for the immunoaffinity purification of phosphodiester α-GlcNAcase to apparent homogeneity. The purification procedure, summarized in Table III, provided a more than 670,000-fold enrichment of phosphodiester α-GlcNAcase specific activity from a bovine liver homogenate with a 14% recovery of enzyme activity. Bovine phosphodiester α-GlcNAcase was purified from frozen calf liver in three steps as described under “Experimental Procedures.” Fetal calf serum was investigated because of Lee and Pierce’s observation that the enzyme was found in human serum (6).  

Properties of Bovine Liver Phosphodiester α-GlcNAcase—When the purified phosphodiester α-GlcNAcase was subjected to SDS-PAGE under either reducing or nonreducing conditions as shown in Fig. 3, it was seen that upon disulfide reduction, the apparent mass of the enzyme changes from approximately 136 to 68 kDa. This behavior is consistent with a dissociation of the enzyme activity solubilized from the membranes was recovered from the UC1-UltraLink™ as shown in Fig. 2A. In contrast, only 0.005% of the input protein was eluted with the enzyme activity by the 0.5 M NaHCO₃, pH 10, buffer. The rest of the protein either did not bind to the affinity matrix or was washed off with the 0.5 M NaHCO₃, pH 8.0. It was important to immediately neutralize the pH 10 eluate, because storage of the highly purified phosphodiester α-GlcNAcase in 0.5 M NaHCO₃, pH 10, caused a slow loss of enzyme activity. The use of 0.5 M NaHCO₃, pH 10, was essential for elution from the UC1-UltraLink™, since the other salts tested (at pH 10) all resulted in prolonged trailing of the eluted phosphodiester α-GlcNAcase. The affinity matrix could be reused a number of times after thorough washing in high salt followed by reequilibration in starting buffer. Although the immunoaffinity step provided a 20,000-fold single step purification, probably due to both the specificity of mAb UC1 and the low nonspecific binding of the UltraLink™ matrix, a final gel filtration step was required to achieve homogeneity. The impurities remaining after immunoaffinity chromatography were removed by the Superose 6 gel filtration step as shown in Fig. 2B. The phosphodiester α-GlcNAcase activity eluted in a single symmetrical peak centered at fraction 30, which corresponded to an apparent molecular mass of 272 kDa based on the mobility of molecular mass standards during gel filtration on the same Superose 6 HR 10/30 column in a separate calibration run. Shown in Fig. 2C is the silver-stained gel obtained when aliquots of the indicated Superose-6 column fractions were subjected to SDS-PAGE under reducing conditions. The staining intensity of the protein band with a mobility of about 68 kDa coincides with the phosphodiester α-GlcNAcase activity.
The bovine liver phosphodiester α-GlcNAcase was previously shown to be a glycoprotein containing N-linked oligosaccharide chains that allow it to bind to concanavalin A-Sepharose (5), but the nature of these N-linked oligosaccharides was unknown. To determine whether the N-linked oligosaccharides of the enzyme were of the complex or high mannose type, the phosphodiester α-GlcNAcase was digested with either PNGase.

**TABLE III**

| Step                  | Volume | Protein concentration | Total protein | Phosphodiester α-GlcNAcase |
|-----------------------|--------|-----------------------|---------------|----------------------------|
|                       | ml     | mg/ml                 | mg            | Activity | Specific activity | Total activity | Yield | Purification |
| Homogenate            | 2,400  | 95                    | 228,000       | 70       | 0.74              | 168,000        | 100   | 1.0          |
| Solubilized membrane  | 1,000  | 14                    | 14,000        | 73.3     | 5.23              | 73,300         | 43.6  | 7.1          |
| UC1-UltraLink™ eluate | 7      | 0.098                 | 0.69          | 9,792    | 99,918            | 68,544         | 40.8  | 135,758      |
| Superose 6 eluate     | 3.75   | 0.012                 | 0.047         | 6,255    | 498,500           | 23,459         | 14    | 673,650      |

**FIG. 2.** A, chromatography of phosphodiester α-GlcNAcase on UC1-UltraLink™. The crude enzyme was bound to 20 ml of UC1-UltraLink™ by incubation for 16 h at 4 °C with constant rotation. The resin was then collected by centrifugation and packed in a 2.6 × 20-cm column and washed at 5 ml/min with buffer E until the absorbance stabilized. The column was then washed with buffer F containing 0.05 M EDTA, and phosphodiester α-GlcNAcase eluted at 2 ml/min with buffer G. Fractions of 1 ml were collected, and an aliquot of each fraction was assayed for protein (●——●) and enzyme activity (○——○). B, chromatography of phosphodiester α-GlcNAcase on Superose 6. Enzyme from UC1-UltraLink™ was concentrated to 0.5 ml by chromatography on POROS-50 HQ and filtration in a Centricon 100 and applied to a 1.0 × 30-cm column of Superose 6. The first 6 ml of effluent were not collected, and then fractions of 0.25 ml were collected and an aliquot of each fraction was assayed for protein (●——●) and phosphodiester α-GlcNAcase activity (○——○). C, SDS-PAGE of phosphodiester α-GlcNAcase in fractions from the Superose 6, run in the presence of reducing agent and stained with silver.
50,000 from the lane 1 (PNGase F-treated) shifted down to approximately 18-kDa mobility shift, suggesting that each monomer may contain as many as 5–7 such oligosaccharide chains depending on their structures. The picture of the enzyme that emerges from these results is one of a tetrameric protein, the 272-kDa form observed on nonreducing gels, which upon reduction of disulfide bonds generates the 68-kDa monomer. In previous work on partially purified phosphodiester \( \alpha \)-GlcNAcase (5), we showed that when the enzyme was subjected to SDS-PAGE without boiling or \( \beta \)-mercaptoethanol in the sample buffer, enzyme activity was recovered in the eluate of gel slices centered at \(-130\ kDa\). No enzyme activity could be recovered from the gel lane if the sample had been reduced with \( \beta \)-mercaptoethanol. Thus, at least in the context of SDS-PAGE, the dimer form of phosphodiester \( \alpha \)-GlcNAcase appears to be the smallest enzymatically active form. It was interesting to discover then, as shown in Fig. 5, that the mAb UC1 only reacted with the nonreduced, dimer form of phosphodiester \( \alpha \)-GlcNAcase on a Western blot and not with the reduced monomer. This observation fits with the fact that mAb UC1 inhibits phosphodiester \( \alpha \)-GlcNAcase activity, presumably by reacting with an epitope in or near the active site of the enzyme. Such an epitope could be destroyed when the dimer is reduced and dissociated into monomers. The monomers appear to be of only one type since amino-terminal sequencing of the homogenous phosphodiester \( \alpha \)-GlcNAcase gave a single unambiguous amino acid sequence as shown under “Experimental Procedures.” Data base searches demonstrate that this is a novel protein (not shown). However, we cannot exclude the possibility that the enzyme contains two different polypeptide subunits of exactly the same molecular weight, each bearing complex-type \( N \)-linked oligosaccharides that contribute 18 kDa in apparent molecular mass, one of which has a blocked amino terminus.

When a rabbit polyclonal antibody was raised to a synthetic peptide corresponding to residues 3–15 of the amino-terminal sequence and affinity-purified on a peptide column, the anti-peptide antibody gave a good signal on Western blots of the purified bovine liver phosphodiester \( \alpha \)-GlcNAcase. As shown in Fig. 6, the blot prepared from a reducing SDS gel of the liver enzyme (Fig. 6A, lane 1) gave a band at 69 kDa and a fainter band at about 140 kDa, which is presumably incompletely.

**Fig. 3.** Phosphodiester \( \alpha \)-GlcNAcase dissociates into monomers upon disulfide reduction. Aliquots of purified phosphodiester \( \alpha \)-GlcNAcase from Superose 6 HR 10/30 column were subjected to electrophoresis on a 4–12% NuPAGE gel, and the protein bands were detected by silver staining as described under “Experimental Procedures.” Left lane, enzyme in the absence of disulfide bond reduction; right lane, enzyme following disulfide bond reduction. The molecular masses of standard proteins electrophoresed on an adjacent lane are indicated to the left.

**Fig. 4.** Glycosidase treatment of purified phosphodiester \( \alpha \)-GlcNAcase. Aliquots of the enzyme (2 \( \mu \)g) of phosphodiester \( \alpha \)-GlcNAcase from the Superose 6 HR10/30 column were subjected to SDS-PAGE as described in the legend to Fig. 3, except that the gel was then transferred to nitrocellulose and probed with mAb UC1 (1:100 diluted ascites) and detected with the secondary antibody rabbit antimouse IgG-labeled with \( ^{125}\)I. The blot was exposed to Biomax film at \(-70^\circ C\) for 30 min. Lane 1, PNGase F treatment; lane 2, PNGase F control; lane 3, Endo Hf treatment, lane 4, Endo Hf control.

**Fig. 5.** Monoclonal antibody UC1 only reacts with nonreduced phosphodiester \( \alpha \)-GlcNAcase on Western blotting. Aliquots (10 \( \mu \)l) of phosphodiester \( \alpha \)-GlcNAcase from the Superose 6 HR10/30 column were subjected to SDS-PAGE as described in the legend to Fig. 3, except that the gel was then transferred to nitrocellulose and probed with mAb UC1 (1:100 diluted ascites) and detected with the secondary antibody rabbit antimouse IgG-labeled with \( ^{125}\)I. The blot was exposed to Biomax film at \(-70^\circ C\) for 30 min. Lane 1, enzyme subjected to disulfide bond reduction; lane 2, enzyme without disulfide bond reduction.
reduced dimer. It is possible to get a good signal with as little as 4–5 units (i.e., approximately 10 ng) of enzyme under the conditions described in the legend to Fig. 6. Unlike the monoclonal antibody UC-1, the polyclonal anti-peptide antibody does not inhibit phosphodiester α-GlcNacase activity when added to assays, nor does it capture enzyme activity onto protein A-Sepharose beads.

Bovine liver phosphodiester α-GlcNacase was analyzed for its content of sialic acid and shown to contain 3.8 mol of sialic acid/mol of monomer. Of this, 25% was N-glycolyl neuraminic acid and 75% was N-acetylsialic acid, which are nicely resolved on the high pressure liquid chromatography column.

Homogenous phosphodiester α-GlcNacase was stable for at least 6 months when stored at −80 °C in 20% glycerol at pH 7.4.

**Properties of Soluble Fetal Calf Serum Phosphodiester α-GlcNacase**—The prior report that phosphodiester α-GlcNacase was present in human serum (6) prompted us to use fetal calf serum as a source for a soluble form of the bovine enzyme. The procedure yielded purified enzyme (about 500 units from 2.5 liters), which eluted from the Superose 6 column with an apparent molecular weight of 270,000. The enzyme was subjected to reducing SDS-PAGE and detected on blots with the anti-clonal antibody UC-1, the polyclonal anti-peptide antibody does not react with the enzyme. Unlike the monomer, the oligomers were removed, and the released [3H]GlcNac was determined by liquid scintillation counting as described previously (10). The pure enzyme released 83% of the [3H]GlcNac as shown in Fig. 7.

**DISCUSSION**

These studies were undertaken with the goal of purifying phosphodiester α-GlcNacase to homogeneity for several purposes: 1) to explore its subunit structure and membrane topology; 2) to obtain peptide sequence for use in cloning the cDNA, which will permit mutagenesis studies to find the structural elements that are responsible for the enzyme's topology in and trafficking to the Golgi membranes; and 3) to provide large amounts of pure phosphodiester α-GlcNacase to use in conjunction with GlcNac-phosphotransferase, to generate mannose 6-phosphate groups on purified lysosomal enzymes that could be used for enzyme replacement studies. Although our earlier studies (5, 13) led us to believe that our "purified" phosphodiester α-GlcNacase was homogenous, we now know that it was only about 1% pure enzyme protein, and the observed protein bands did not represent phosphodiester α-GlcNacase. Attempts to further purify the previous preparation using affinity chromatography with several substrate analog columns were unsuccessful (data not shown), so as an alternative strategy the immunoaffinity approach described here was undertaken.

The strategy employed in the current study to identify monoclonal antibodies specific for phosphodiester α-GlcNacase may...
be generally applicable to the isolation of other enzymes where conventional purification techniques are unsuccessful. This approach requires only a partially purified enzyme and a specific enzyme assay, requirements that can be met for many enzymes that have yet to be isolated. Interestingly, mAb UC1 markedly inhibits phosphodiester α-GlcNAcase activity. Despite this, the functional screen that required capture of enzyme activity successfully identified the UC1-secreting hybridoma. This may be because phosphodiester α-GlcNAcase is a complex of four subunits arranged as a pair of dimers, each of which is expected to contain at least one active site. Whether this approach can be applied to monomeric enzymes remains to be demonstrated.

Having the pure homogenous phosphodiester α-GlcNAcase in hand has allowed us to answer a number of questions about the enzyme that could not be addressed with only partially purified enzyme. In regard to its subunit structure, phosphodiester α-GlcNAcase in both its membrane and soluble forms has been shown to exist as a homotetramer composed of two dimers, each containing a pair of disulfide-linked monomers. Since the monomer has no discernible enzyme activity, it had escaped detection earlier (5, 13).

The fact that the rabbit polyclonal antibody directed to the amino-terminal peptide of the bovine liver phosphodiester α-GlcNAcase (membrane form) also reacts with the soluble enzyme from fetal calf serum suggests that the phosphodiester α-GlcNAcase is likely to be a type I membrane-spanning glycoprotein with its amino terminus in the lumen of the Golgi apparatus. It is not uncommon for a small proportion of membrane proteins of the secretory pathway of liver to undergo a proteolytic clip in the luminal domain and be secreted into the blood stream. For example, a soluble form of Galβ1–4GlcNAc α-2,6-sialyltransferase can be released from rat liver Golgi by a cathepsin D-like protease activity located at the luminal face of the Golgi (14). Interestingly, the topology for the phosphodiester α-GlcNAcase (NH₂ terminus in the lumen) is opposite to the orientation of the known Golgi trimming mannosidases and the glycosyl transferases, which to date are all type II membrane-spanning proteins (15).

Finally, the finding that bovine liver phosphodiester α-GlcNAcase contains complex N-linked oligosaccharides with appreciable amounts of sialic acid indicates that at least a portion of the phosphodiester α-GlcNAcase, a cis-Golgi-acting enzyme, must travel to the trans-Golgi network, where sialyl transferase resides. This indicates that phosphodiester α-GlcNAcase may traverse a more complex intracellular itinerary than simply arriving at the cis-Golgi from its site of synthesis in the endoplasmic reticulum. Cloning of the enzyme, which is under way, will allow us to express the phosphodiester α-GlcNAcase and test what regions of the molecule are involved in its intracellular trafficking.

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