Evidence for the Involvement of Glu-355 in the Catalytic Action of Human β-Hexosaminidase B*

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In a previous study the photoactivable affinity probe, 3-azi-1-[((6-^{3}H)2-acetamido-2-deoxy-1-β-D-galactopyranosyl)thio]-butane, was used to identify the active site of β-hexosaminidase B, a β-subunit dimer (Liessem, B., Glombitza, G. J., Knoll, F., Lehmann, J., Kellermann, J., Lottspeich, F., and Sandhoff, K. (1995) *J. Biol. Chem.* 270, 23693–23699). The probe predominately labeled Glu-355, a highly conserved residue among hexosaminidases. To determine if Glu-355 has a role in catalysis, β-subunit mutants were prepared with the Glu-355 codon altered to either Ala, Gln, Asp, or Trp. After expression of mutant proteins using recombinant baculovirus, the enzyme activity associated with the β-subunits was found to be reduced to background levels. Although catalytic activity was lost, the mutations did not otherwise affect the folding or assembly of the subunits. The mutant β-subunits could be isolated using substrate affinity chromatography, indicating they contained intact substrate binding sites. As shown by cross-linking with disuccinimidyl suberate, the mutant β-subunits were properly assembled. They could also participate in the formation of functional β-hexosaminidase A activity as indicated by activator-dependent G(M2) ganglioside degradation activity produced by co-expression of the mutant β-subunits with the α-subunit. Finally, the mutant subunits showed normal lysosomal processing in COS-1 cells, demonstrating that a transport-competent protein conformation had been attained. Collectively the results provide strong support for the intimate involvement of Glu-355 in β-hexosaminidase B-mediated catalysis.

The human lysosomal β-hexosaminidases (EC 3.2.1.52) are composed of two structurally related subunits, α and β (for reviews see Refs. 1 and 2). After synthesis, the precursor subunits dimerize and form three isozymes: β-hexosaminidase A (αβ), β-hexosaminidase B (ββ), and β-hexosaminidase S (αα). Proper folding and assembly of the subunits is required for expression of catalytic activity and for transport of the enzymes out of the endoplasmic reticulum (3, 4). After reaching their final destination within lysosomes, the precursor polypeptides undergo proteolytic processing to a mature lysosomal form.

The β-hexosaminidases degrade glycoproteins, glycolipids, and proteoglycans through the release of terminal β-glycosidically linked N-acetylglucosamine or N-acetylgalactosamine residues. Each subunit possesses an active site within the context of the dimeric structure of the holoenzyme. The α and β active sites, although functionally very similar, exhibit differences in their ability to hydrolyze sulfated substrates (5). Significant substrate specificity differences also exist between the isozymes. Most notably, only the heterodimer β-hexosaminidase A together with the G_{M2}[1] activator protein is able to degrade G_{M2} ganglioside at significant rates (6). The G_{M2} activator protein functions through binding the ganglioside substrate and interacting with β-hexosaminidase A. The release of the terminal N-acetylgalactosamine residue from the ganglioside is accomplished via the α-chain active site (5, 7, 8). However, the β-subunit contributes essential functions in this reaction, possibly in promoting the interaction with the activator protein (9).

Mutations in the *HEXA* (α-subunit) and *HEXB* (β-subunit) genes cause Tay-Sachs and Sandhoff diseases, respectively. Defects in the *GM2A* gene result in G_{M2} activator deficiency. In each of these genetic disorders there is a massive accumulation of G_{M2} ganglioside and related glycolipids in neuronal lysosomes, leading to severe neurodegeneration. In addition to G_{M2} ganglioside and related glycolipids, glycosaminoglycans are also critical substrates as demonstrated by the severe mucopolysaccharidoses phenotype exhibited in mice lacking both subunits of hexosaminidase (10).

Although much progress has been made in understanding the genetics and biochemistry of the hexosaminidases, details concerning the structures of the active sites and mechanisms of catalysis have only recently emerged. The first biochemical evidence localizing an active site was presented in a study by Liessem *et al.* (11) using the photoaffinity reagent, [3H]ATB-GalNAc. This probe was shown to label Glu-355 of the human β-subunit, implicating this amino acid in the architecture of the active site. To determine if Glu-355 has a role in β-hex-

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[1] The abbreviations used are: G_{M2} ganglioside, GalNAcβ1→4-(NeuAcα2→3)Galβ1→4Glcβ1→1Cer; *HEXA*, the gene encoding the α-subunit of β-hexosaminidase; *HEXB*, the gene encoding the β-subunit of β-hexosaminidase; *GM2A*, the gene encoding the G_{M2} activator protein; MU-GlcNAc, 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside; MU-GlcNAc-6-SO_4, 4-methylumbelliferyl-6-sulfo-2-acetamido-2-deoxy-β-D-glucopyranoside; [3H]ATB-GalNAc, 3-azi-1-[((6-^{3}H)2-acetamido-2-deoxy-1-β-D-galactopyranosyl)thio]-butane; DSS, disuccinimidyl suberate; δ-lactone, 2-acetamido-2-deoxy-β-D-glucono-1,5-lactone; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

osaminidase B-mediated catalysis, we altered this residue by site-directed mutagenesis. The mutant β-subunits lost catalytic activity but retained their ability to bind a substrate analogue, form homo- and heterodimers, function in the context of β-hexosaminidase A in activator-dependent G_{M2} degradation, and undergo intracellular transport to lysosomes. These results, together with affinity labeling of Glu-355 and the recently described structure of an evolutionarily related chitobiase (12), provide compelling evidence that Glu-355 has an essential role in the catalytic mechanism of human β-hexosaminidase B.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—The mutations were made with the Sculptor *in vitro* mutagenesis system from Amersham Corp. (RPN 1526) using the human β-subunit cDNA that had been cloned into M13mp18 (13). The mutant oligonucleotides (antisense) were as follows: E355A, CCCAACATTTAAATTCCAC<u>TGC</u>ATCTCCTCCCAAATG; E355Q, CCCAACATTTAAATTCCAC<u>TTG</u>ATCTCCTCCCAAATG; E355D, CCCAACATTTAAATTCCAC<u>ATC</u>ATCTCCTCCCAAATG; E355W, CCCAACATTTAAATTCCAC<u>TGC</u>ATCTCCTCCCAAATG.

The resultant mutant cDNAs were cloned into the *Xba*I and *Sac*I sites of the pSVL vector (Pharmacia Biotech Inc.) for expression in COS-1 cells. For the production of baculovirus, the mutant cDNAs were excised from the pSVL vector and subcloned into the *Xba*I/*Sma*I sites of the baculovirus shuttle vector pVL1392 (Pharmingen). All mutant constructs were verified by DNA sequencing.

Expression of Mutant Constructs—Clonal recombinant baculovirus containing each of the mutant cDNAs was produced as described except that the virus producing the mutant cDNAs was identified by production of immunoreactive β-subunits rather than by enzymatic activity (9). The recombinant baculovirus containing wild-type β-subunit was described previously (14). Production of expression media by infection of insect cells (High Five and SF21) and co-expression of the mutant β-subunit cDNAs with the α-subunit was accomplished as described by Pennybacker *et al.* (9). COS-1 cells were transfected with mutant cDNAs in the pSVL vector using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's instructions. [^{35}S]Methionine labeling and immunoprecipitation have been described (13).

Assay of Mutant Proteins—Enzyme activity was determined with MU-GlcNAc and MU-GlcNAc-6-SO$_4$ as described previously (9). Protein concentrations were measured according to the method of Bradford (15) using bovine serum albumin as a standard. [^{3}H]G_{M2} ganglioside, labeled in the GalNAc moiety, was used to determine G_{M2} degrading activity in the presence of recombinant G_{M2} activator protein (9).

Affinity Chromatography—The affinity gel was prepared as described by Izumi and Suzuki (16) using 2-acetamido-*N*-(ε-aminocaproyl)-2-deoxy-β-D-glucopranosylamine as an affinity ligand. Small columns were prepared with a 1-ml bed volume and were equilibrated in 10 mM phosphate buffer (pH 6.0) containing 200 mM NaCl. Samples (0.5 ml) of dialyzed and concentrated expression medium were loaded on the affinity columns. Nonspecifically bound protein was removed with 2.5 ml of 10 mM phosphate buffer (pH 6.0) containing 100 mM NaCl. Specifically bound proteins were eluted with 2 ml of 150 μM δ-lactone in equilibration buffer. Proteins were analyzed by 10% Tricine polyacrylamide gels as described by Schägger and von Jagow (17). In the case of E355D, wash-through and eluate were concentrated approximately 5-fold before electrophoresis. Protein bands were visualized by silver staining as described by Blum *et al.* (18). For Western blotting, equal volumes of eluate were resolved by SDS-polyacrylamide gel electrophoresis as described above. Proteins were transferred to polyvinylidene difluoride membrane. The membranes were blocked overnight at 4 °C and then incubated with goat anti-hexosaminidase B for 1.5 h at room temperature. The membranes were washed with Tris buffer (40 mM Tris-HCl, 340 mM NaCl, 0.1% Nonidet P-40, 0.01% NaN$_3$, pH 7.4). After 1 h of incubation with rabbit anti-goat IgG coupled to alkaline phosphatase followed by washing in Tris buffer, the membrane was developed using 0.7 mM 5-bromo-4-chloro-3-indolyl phosphate and 0.2 mM nitro blue tetrazolium in carbonate buffer (100 mM carbonate, 1 mM MgCl$_2$, 0.01% NaN$_3$, pH 9.8).

Cross-linking—Disuccinimidyl suberate (DSS, 10 mM) was freshly prepared in dimethyl sulfoxide. β-Subunit preparations (30 ng in 75 mM phosphate buffer, pH 7.5) were incubated in the presence of 0.78 mM DSS at 37 °C for 30 min. Control samples contained an equivalent amount of dimethyl sulfoxide without the cross-linker. The reaction was stopped by the addition of Tris-HCl (pH 7.5) to 27 mM. The samples
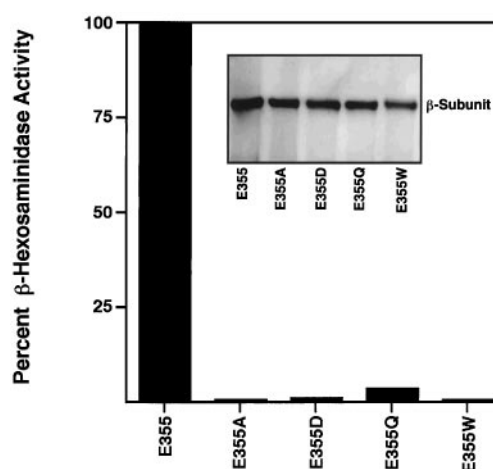

FIG. 1. **Enzyme activity of Glu-355 (*E355*) β-subunit mutants.** Cultures of insect cells were infected with recombinant baculovirus containing the unaltered β-subunit (Glu-355 (*E355*)) or β-subunit mutants (*E355Q, E355A, E355W, E355D*). A portion of each expression medium was used to determine hexosaminidase activity with the synthetic substrate, MU-GlcNAc. Another portion was subjected to Western analysis with antibody to β-hexosaminidase B (*inset*). The probed membrane was scanned with a Molecular Dynamics Storm 860 Fluorimager, and the relative amount of β-subunit in each sample was determined with Image Quant 1.1 software. The activity of each sample was normalized to the amount of β-subunit protein. The wild-type β-subunit (Glu-355) was considered to express 100% activity. Determination was carried out in duplicate, and the deviation did not exceed 5%.

were electrophoresed on an SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. After probing the membrane with anti-hexosaminidase B, detection was accomplished with the Vistra ECF Western blotting kit (Amersham). Probed membranes were scanned with the Storm 860 Fluorimager from Molecular Dynamics.

RESULTS

The codon specifying Glu-355 in the β-hexosaminidase β-subunit cDNA was altered to produce cDNAs with the following changes: E355A, E355W, E355Q, and E355D. The cDNAs were recombined into baculovirus for expression in insect cells. We previously showed that precursor enzyme can be recovered from the expression medium for analysis in a number of biochemical assays (9). The expression medium from cells infected with each recombinant virus was assayed for enzymatic activity with the synthetic substrate, MU-GlcNAc, and for β-subunit protein by Western blotting (Fig. 1). Abundant β-hexosaminidase activity was detected in medium from insect cells infected with virus expressing the unaltered β-subunit (Glu-355). In contrast, expression media containing the Glu-355 mutant β-subunits resulting from infection with baculovirus carrying any of the mutant cDNAs (E355A, E355Q, E355W, E355D) displayed very low levels of activity (≤3%) relative to wild-type β-hexosaminidase B after normalization to the amount of β-subunit protein present.

The expression media were passed through miniaffinity columns containing the ligand, 2-acetamido-*N*-(ε-aminocaproyl)-2-deoxy-β-D-glucopranosylamine (Fig. 2). This is the classical affinity ligand used for purification of β-hexosaminidase via its substrate binding site (16, 19, 20). After washing the columns, elution was carried out with the high affinity competitive inhibitor, δ-lactone, a transition state analogue with a submicromolar K_i. If the mutant subunits retained their substrate binding sites, they should bind to the columns and then be eluted specifically with δ-lactone. The wash-through fraction obtained from each expression medium showed a complex mixture of protein species after electrophoresis on SDS-gels and visualization by silver staining. As expected, a M_r 63,000 polypeptide, the precursor size of the β-subunit (20), was specifically eluted
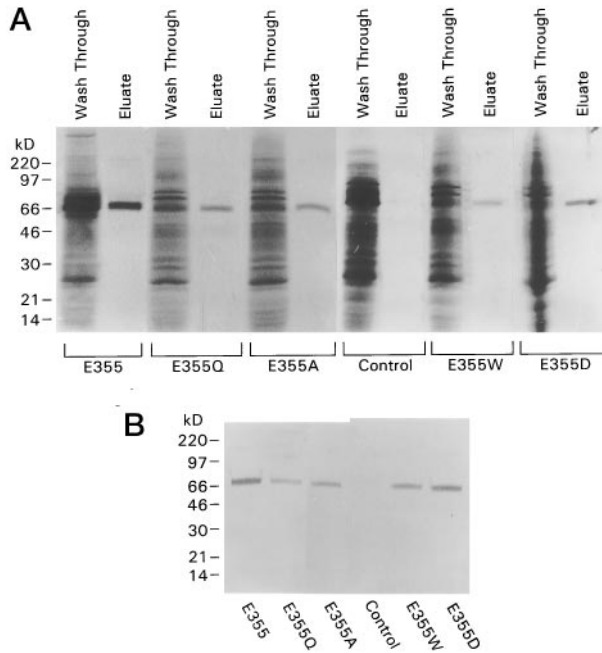
FIG. 2. **Purification of β-subunit Glu-355 (E355) mutants by substrate affinity chromatography.** Expression media prepared by infection of insect cells with baculovirus containing the unaltered β-subunit (Glu-355 (*E355*)), β-subunit mutants (*E355Q, E355A, E355W, E355D*), and the wild-type baculovirus (*Control*) were passed through mini-columns of 2-acetamido-*N*-(ε-aminocaproyl)-2-deoxy-β-D-glucopyranosylamine coupled to Sepharose. The wash-through was collected. After rinsing the column, elution was carried out with 150 μM δ-lactone. Samples of the wash-through and elution were electrophoresed on an SDS-polyacrylamide gel. The gels were then silver-stained to reveal proteins (*panel A*). Portions of the elution fractions were subjected to Western analysis using antibody directed at β-hexosaminidase B (*panel B*).

TABLE I
Kₘ and V_max values of recombinant β-hexosaminidase β-subunit preparations purified by substrate affinity chromatography

Measurements were made with MU-GlcNAc. Glu-355 (E355) is the unaltered β-subunit. Control refers to the preparation obtained by infection of insect cells with the wild-type baculovirus and represents the endogenous insect cell activity.

Enzyme	$K_m{}^a$	$V_{max}{}^{a,b}$
	mM	*μmol/min/mg*
β-Hexosaminidase A (placental)	0.70	138
β-Hexosaminidase B (placental)	0.57	250
E355	0.63	174
E355Q	0.40	5.5
E355A	0.63	9.7
E355W	0.63	8.6
E355D	0.59	6.5
Control	0.56	8.9

[a] Determinations were carried out in duplicate; deviation did not exceed 5%. K_m and V_{max} values were calculated from double-reciprocal plots of reaction velocity *versus* substrate concentration.

[b] V_{max} values were calculated from protein concentrations determined by the Bradford method. Due to the low amount of protein recovered from the affinity column, the mutant preparation V_{max} values have an experimental error of ≤50%.
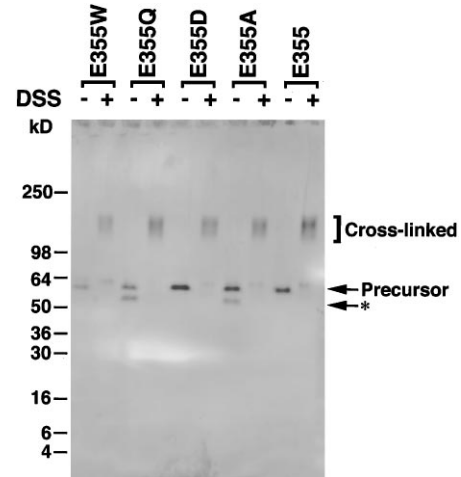

FIG. 3. **Cross-linking of β-subunit Glu-355 (E355) mutants.** Samples of baculovirus expressed β-hexosaminidase B (Glu-355 (*E355*)) and β-subunit mutants (*E355Q, E355A, E355W, E355D*) were incubated with or without DSS. The samples were electrophoresed on an SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and subjected to Western analysis using antibody directed at β-hexosaminidase B. The protein band marked by the *asterisk* appears after the incubation at 37 °C and likely represents a proteolytic breakdown product.

from the affinity column that had been loaded with expression medium containing β-hexosaminidase B (Glu-355 *(E355)*, Fig. 2*A*). Similar results were obtained with the expression media obtained by infection with virus carrying the mutant β-subunit cDNAs even though only minimal enzyme activity had been found. In each case the M_r 63,000 polypeptide was proven to be the hexosaminidase β-subunit by virtue of its reactivity with antiserum prepared against β-hexosaminidase B (Fig. 2*B*). No comparable peptide was found in the medium from cells infected with wild-type virus (control).

The affinity-purified preparations were used for determination of kinetic parameters with the synthetic substrate, MU-GlcNAc (Table I). The recombinant hexosaminidase B displayed a V_{max} and K_m similar to the purified placental enzymes. A low level of activity was detected in the preparation derived from the wild-type virus infection (*control*). This background activity likely represents a previously described insect cell activity released during viral infection (21) that apparently binds to the affinity column. The V_{max} values of each of the affinity-purified mutant preparations were reduced to near the background level of the control virus preparation. The K_m values were also close to that of the background insect cell enzyme and may be the result of interference by this endogenous activity.

The mutant subunits were tested to ensure their structural and functional integrity in light of their catalytic deficiency. First, mutant β-subunit preparations were cross-linked with DSS to determine if they had assembled, a state required for enzymatic activity to be expressed (4). As seen in Fig. 3, the catalytically impaired mutant proteins were indistinguishable from catalytically active β-hexosaminidase B (Glu-355) in their

capacity to be cross-linked by DSS.

Next, we tested whether the catalytically deficient mutant β-subunits could function in the context of the heterodimer β-hexosaminidase A in activator-dependent G_{M2} ganglioside degradation. The α-subunit was co-expressed with each β-subunit to produce heterodimers. The resulting expression media were assayed for the capacity to hydrolyze labeled G_{M2} ganglioside in the presence of activator protein. All mutant β-chains, in concert with the α-subunit, were competent in their capacity to degrade the ganglioside (Fig. 4). The G_{M2} ganglioside-degrading activities of the expression media containing the mutant heterodimers ranged from 77 to 120% of the activity of co-expressed wild-type α- and β-subunits (Fig. 4).

Finally, the mutant subunits were expressed in COS-1 cells to determine their intracellular fate (Fig. 5). After transfection, the mutant constructs did not produce enzyme activity above background levels (not shown). However, the mutant constructs did direct the production of M_r 63,000 β-subunit pre-
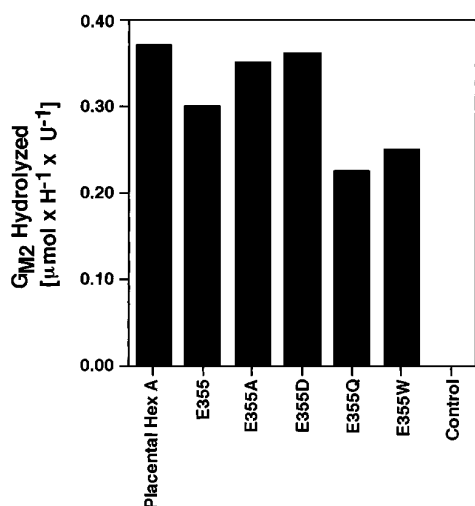
FIG. 4. **Activator-dependent hydrolysis of G_{M2} ganglioside by heterodimers of the α-subunit and β-subunit Glu-355 (*E355*) mutants.** Insect cells were co-infected with recombinant baculovirus containing α-subunit together with the unaltered β-subunit or mutant β-subunits (*E355Q, E355A, E355W, E355D*). The *Control* sample was from an infection with wild-type baculovirus. The expressed enzyme in the culture media was assayed for degradation of [³H]G_{M2} ganglioside in the presence of recombinant G_{M2} activator protein. The activity is expressed as μmol of G_{M2} ganglioside cleaved/h/unit of MU-GlcNAc-6-SO_4 activity. Experiments were carried out in duplicate, and deviations did not exceed 10%.

FIG. 5. **Lysosomal processing of β-subunit Glu-355 (*E355*) mutants in COS-1 cells.** The unaltered β-subunit (Glu-355 (*E355*), mutant β-subunits (*E355Q, E355A, E355W, E355D*), and the empty pSVL vector were transfected into duplicate sets of COS-1 cells as indicated. After 48 h the cells were incubated with [³⁵S]methionine for 2 h. One set of cultures was harvested (*Pulse*). The other was incubated further with unlabeled growth medium for 18 h before harvesting (*Chase*). The extracts of the radiolabeled cells were subjected to immunoprecipitation with antibody directed at β-hexosaminidase B followed by SDS-polyacrylamide gel electrophoresis.

cursors (Fig. 5, *Pulse*) that were converted to the mature, lysosomal forms ($β_a$-chain, M_r 29,000 and $β_b$-chains, M_r 24,000) upon chase in a manner indistinguishable from the unaltered β-subunit (Glu-355) (Fig. 5, *Chase*) (13).

DISCUSSION

Using [³H]ATB-GalNAc as a photoaffinity label for the substrate binding site of β-hexosaminidase B, Liessem *et al.* (11) showed a specific incorporation into Glu-355 of the β-subunit. This result, the strongest biochemical evidence to date in the identification of an active site, focused our attention on this evolutionarily conserved amino acid. We have now extended these studies by analyzing mutant β-subunits with Glu-355 converted into four other amino acids, Ala, Asp, Trp, and Gln. Each of the mutant proteins showed an abrogation of enzymatic activity to near background levels. Despite the catalytic deficit, other functions and properties associated with the β-subunit were normal, indicating that the mutations did not adversely affect the folding and quaternary structure of the protein.

The mutant β-chains underwent normal proteolytic maturation in COS-1 cells indicative of lysosomal delivery. The capacity to pass through the endoplasmic reticulum "quality control" system is evidence for the correct folding and assembly of subunits (22). Mutations in hexosaminidase subunits impairing their folding and assembly leads to premature degradation before lysosome delivery (2). We also directly demonstrated that the subunits were in an assembled state. The mutant β-subunits were indistinguishable from wild-type β-subunits in their capacity to be chemically cross-linked. Activator-dependent G_{M2} ganglioside degradation, a functional assay for heterodimer formation with the α-subunit, was also found to be unimpaired. Since ganglioside degradation is mediated through the α-subunit active site, it would be expected that a defective β active site should not affect hydrolysis. However, the presence of the β-subunit is essential for ganglioside hydrolysis, perhaps by contributing to the interaction with the activator-ganglioside complex. It is clear that the presence of the Glu-355 mutations did not impair these essential functions.
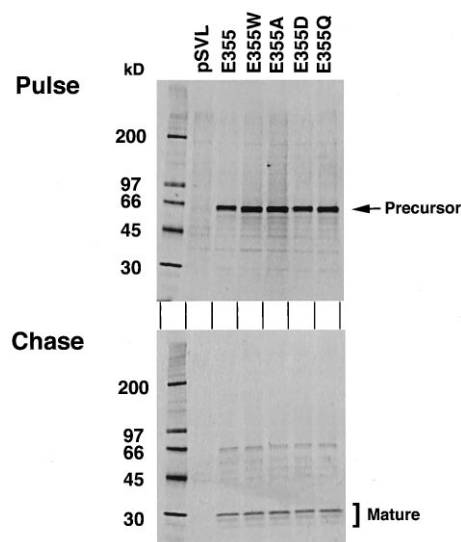
We found that the mutant proteins could be isolated using a substrate affinity column. This result indicates that the substrate binding site on the mutant proteins is sufficiently intact for interaction with the low affinity ligand, 2-acetamido-*N*-(ε-aminocaproyl)-2-deoxy-β-D-glucopranosylamine (K_i ~0.5 mM). Mutant proteins that were affinity-isolated through their substrate binding site were catalytically impaired. The presence of substrate binding in association with a large decrease in enzymatic activity is, in itself, strong evidence for the involvement of Glu-355 in the catalytic mechanism of β-hexosaminidase B. Due to the high degree of sequence similarity between the α- and β-subunits and the functional similarities of their active sites, it is expected that α-subunit Glu-323, the counterpart of β-subunit Glu-355, would also be involved in catalysis mediated by the α-subunit. Mutagenesis of α-subunit Glu-323 also impairs catalytic activity (not shown).

Recently, the crystal structure of *Serratia marcescens* chitobiase, a member of the glycosyl hydrolase family that also includes the human β-hexosaminidases, was determined (12). Based on the structure of the enzyme-substrate complex, an acid-base substrate-assisted catalytic mechanism was proposed in which Glu-540 of the chitobiase functions as the catalytic acid while the polar acetamido group of the sugar substrate serves as a nucleophile in the reaction. Homology modeling of the catalytic domains of the human enzymes suggests that they adhere to the same catalytic mechanism as proposed for chitobiase (12). Significantly, sequence alignments show that Glu-540, the proposed proton donor in chitobiase, coincides with Glu-323 of human α-subunit, the equivalent of Glu-355 in the human β-subunit. Our results, in combination with the structure of the related chitobiase, would indicate that Glu-355 serves as the proton donor during the catalytic action of β-hexosaminidase B.

Previously, two other amino acids, Arg-211 and Asp-196, were suggested to be involved in the β-hexosaminidase B catalytic mechanism (23, 24). However, current evidence does not support a direct role for either of these amino acids in this process. The structure of chitobiase indicates that the counter-

part of Arg-211 is involved in substrate binding and not in the catalytic mechanism (12). Asp-196 was suggested to serve as the acid catalyst in β-hexosaminidase B (24). This seems very unlikely given the very strong evidence summarized here supporting Glu-355 for this role and because all proton donors in glycosidases analyzed thus far have been found to be glutamic acid (25).

In conclusion, three independent lines of evidence provide compelling support for the involvement of Glu-355 in the catalytic mechanism mediated by β-hexosaminidase B. First, Glu-355 was uniquely labeled by an active-site affinity probe. Second, the structure of an evolutionarily related enzyme suggests that the counterpart of Glu-355 functions as the catalytic acid in an acid-base enzyme mechanism. Third, the data presented in this report show that mutant β-subunits with Glu-355 altered to any of four other amino acids display minimal β-hexosaminidase B activity even though other properties of the β-subunit, including formation of the substrate binding site, subunit assembly, activator-dependent G_{M2} degradation by β-hexosaminidase A, and intracellular transport, remain intact.

Note Added in Proof—After this manuscript was accepted for publication, Fernandes *et al.* (26) reported that mutagenesis of Glu-323 of the α-subunit, the equivalent of Glu-355 of the β-subunit, impairs catalytic activity, supporting our conclusions.

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