The carbene precursor 3-azi-1-\(^{[6-3H]}\)-2-acetamido-2-deoxy-1-β-D-galactopyranosyl(thio)butane (also designated \(^{[6-3H]}\)-1-ATB-GalNAc) has been used as a photoaffinity label for human lysosomal β-hexosaminidase B (Hex B, EC 3.2.1.52) purified from postmortal liver. \(^{[3H]}\)-1-ATB-GalNAc behaved as an active site-directed inhibitor, which bound covalently to Hex B upon photolysis at 350 nm and resulted in 15% inactivation of enzyme activity. Up to 75% of the inactivation of Hex B was prevented by including the competitive inhibitor 2-acetamido-2-deoxy-β-D-glucono-1,5-lactone in the photoaffinity experiment. Incubation of \(^{[6-3H]}\)-1-ATB-GalNAc with the enzyme followed by irradiation and subsequent separation of the three polypeptides composing the β-subunit led mainly to labeling of the β-α-polypeptide. Subsequent proteolysis of β, with trypsin and separation of the resulting peptides by high pressure liquid chromatography yielded one prominently labeled peptide fraction. Edman degradation resulted in the sequence E339ISEVFPDQFIHLGGD-EVEFK359. However, no modified amino acid was detected, indicating that the photoaffinity label was presumably bound to the peptide by a labile ester linkage. This was proven when the radiolabel was almost completely released from the peptide by treatment with aqueous ammonium hydroxide. Simultaneously, Glu-355 was converted into Gln-355, which is located within a region of Hex B that shows considerable homology with the α-subunit of human hexosaminidase A and other hexosaminidases from various species.

Human lysosomal hexosaminidases (EC 3.2.1.52) release terminal β-glycosidically linked N-acetylglucosamine and N-acetyl-galactosamine residues from a number of glycoconjugates. They are involved in the intracellular degradation of glycolipids, like ganglioside GM2, its asialo derivative, and globoside, as well as in the degradation of carbohydrate chains of glycoproteins, glycosaminoglycans, and oligosaccharides (for review see Refs. 1 and 2).

The major isoenzymes β-hexosaminidase A and β-hexosaminidase B (Hex B)\(^{2}\) are composed of two noncovalently linked subunits of nearly equal molecular mass. β-Hexosaminidase A is a heterodimer (αβ), whereas Hex B is a homodimer of two β-chains (ββ) (3). The homodimer αβ, also called β-hexosaminidase S, has been detected in patients with Sandhoff’s disease and seems to be unstable under normal conditions (4, 5). The mature α-chain is composed of a major α-polypeptide (M\(_{\text{r}}\) = ~54,000) and a minor polypeptide, M\(_{\text{r}}\) (M\(_{\text{r}}\) = ~6,000). The mature β-subunit originates from its precursor by two proteolytic cleavage events forming a small β\(_{\text{α}}\)-polypeptide (M\(_{\text{r}}\) = ~11,000), the β\(_{\text{β}}\)-chain (M\(_{\text{r}}\) = ~24,000), and the β\(_{\text{α}}\)-chain (M\(_{\text{r}}\) = ~28,000). In the mature β-subunit all 3 chain components are linked by disulfide bonds (6–10). Dimerization of the subunits α and β or β and β is essential for catalytic activity of human β-hexosaminidase A and B, respectively (11). Each subunit possesses an active site that differs in its substrate specificity.

The active site of the β-chain hydrolyzes charged substrates, whereas the α-subunit, in addition, slowly cleaves negatively charged substrates (12). However, only β-hexosaminidase A is able to hydrolyze the most important natural lipid-substrate, ganglioside GM2, in presence of the GM2 activator protein at significant rates (13).

Mutations in any of the three genes encoding for the α- or β-subunit or the GM2 activator protein can give rise to neuronal storage of ganglioside GM2 in one of the three variants of GM2 gangliosidoses. In variant B (Tay-Sachs disease), deficiency of the α-subunit caused by mutations of the HEXA gene brings about a lack of β-hexosaminidase A and β-hexosaminidase S activities, whereas the activity of Hex B is normal or increased. Variant 0 (Sandhoff’s disease) is characterized by β-hexosaminidase A and B deficiency due to mutations of the HEXB gene encoding the β-subunit. In variant AB the GM2 activator protein is deficient due to mutations of the GMA2 gene, whereas β-hexosaminidase A and B activity levels appear to be normal (for review see Ref. 14).

Although human hexosaminidases have been studied intensively, including their gene structures, peptide sequences, biosynthesis, and post-translational modifications, their active sites remain unknown. Moreover, although Hex B has been

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\(^{2}\) The abbreviations used are: Hex B, β-hexosaminidase B; glycolipid GM\(_{2}\), GalNAc\(_{a}\); GalNAc\(_{b}\); GaI\(_{a}\); 4-Gal\(_{a}\); 4-Gal\(_{b}\); 1-Cer; ganglioside GM\(_{2}\), GalNAc\(_{a}\); GalNAc\(_{b}\); GaI\(_{a}\); 4-Gal\(_{a}\); 4-Gal\(_{b}\); 1-Cer; \(^{[6-3H]}\)-1-ATB-GalNAc, 3-azi-1-\(^{[6-3H]}\)-2-acetamido-2-deoxy-1-β-D-galactopyranosyl(thio)butane; 6-lactone, 2-acetamido-2-deoxy-β-D-glucono-1,5-lactone; MUG, 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside; MUGS, 4-methylumbelliferyl-6-sulfon-2-acetamido-2-deoxy-β-D-glucopyranoside; HPLC, high performance liquid chromatography; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin.

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\(^{1}\) The terminology used for gangliosides is that recommended by Svennerholm (18).
crystallized (15), no crystallographic structure of a hexosaminidase is available from which sequence alignments and modeling might be possible. Given the related substrate specificities of the α- and β-subunits and the considerable extent of sequence homology between them, it seems likely that their active sites have a high degree of structural similarity. Site-directed mutagenesis techniques and naturally occurring mutations yield useful information for the identification of amino acids involved, but interpretation of such studies is difficult in the absence of either crystallographic or affinity labeling information.

Therefore, we studied the binding site of Hex B using active site-directed labeling techniques employing [3H]-1-ATB-GalNAc as a photoaffinity label for the purified enzyme. The chemical structure of this ligand is shown in Fig. 1. Because glycoside hydrolases usually show a high glycan-specificity and a relatively low aglycon-specificity, the photolabile group was introduced into the aglycon of the enzyme-resistant thioglycoside so that recognition of the compound as a substrate analogue was not impaired (16). After photolysis at 350 nm, the diazirine group located in the aglycon forms a highly reactive carbene, which can react covalently with amino acid side chains. [3H]-1-ATB-GalNAc has been previously shown to specifically bind at the substrate binding site of β-hexosaminidase A (17). The K\textsubscript{d} values of this compound were estimated to be 1.8 \textmu M and 3.0 \textmu M for the β-subunit of β-hexosaminidase A and B, respectively.

The present study includes specific photoincorporation of [3H]-1-ATB-GalNAc into purified human lysosomal Hex B, tryptic digestion of the covalently labeled enzyme, and isolation and sequencing of the labeled peptide.

EXPERIMENTAL PROCEDURES

Chemicals—MUG, MUGS, and δ-lactone were purchased from Toronto Research Chemicals (Toronto, Canada). Methyl α-β-glucopyranoside and concanavalin A-Sepharose CL-4B were from Sigma (Deisenhofen, Germany). HPLC grade water, isopropanol, and acetonitrile were obtained from J. T. Baker (Deventer, Holland). Sequencing grade modified trypsin (reductively methylated and sequenced) and concanavalin A-Sepharose CL-4B were purchased from Dako (Carpinteria, CA). 1,2-Diiodoethane was purchased from J. T. Baker (Deventer, Holland). HPLC grade water, isopropanol, and acetonitrile were obtained from J. T. Baker (Deventer, Holland). 1,2-Diiodoethane was purchased from J. T. Baker (Deventer, Holland).

Purification of Lysosomal β-Hexosaminidase B—Isolation of Hex B from human liver was performed as described previously (20) and modified using fast protein liquid chromatography techniques. All buffers contained 0.01% NaN\textsubscript{3} (w/v) as preservative. Brieﬂy, liver extract were brought to 70% saturation with (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}. The precipitated proteins were dissolved in phosphate buffer (50 mM, pH 7.0) and loaded onto a concanavalin A-Sepharose 4B column. Nonspecifically bound protein was washed away with the same buffer containing 200 mM NaCl. Bound glycoproteins were eluted using the same buffer containing 10% (w/v) methyl α-β-glucopyranoside. Fractions with hexosaminidase activity were dialyzed against phosphate buffer (10 mM, pH 6.0) and loaded onto a Q-Sepharose column, which was connected to a fast protein liquid chromatography system. Hex B, which elutes with the void volume, was subsequently loaded onto a S-Sepharose column and eluted with a linear gradient of 0–0.5 M NaCl. Fractions with Hex B activity were concentrated and dialyzed against phosphate buffer (10 mM, pH 7.0, containing 150 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}) and loaded onto a Superdex 200 gel filtration column (Q-Sepharose-Hiload 16/10, S-Sepharose-Hiload 16/10, Superdex 200-Hiload 16/60, and the fast protein liquid chromatography system were from Pharmacia, Uppsala, Sweden). Enzyme activity was assayed during each step of purification. Protein was detected by continuous monitoring at 280 nm. Homogeneity of the enzyme was assessed by SDS-polyacrylamide gel electrophoresis.

Photoaffinity Labeling of Hex B and Determination of Inactivation—385 μCi of [3H]-1-ATB-GalNAc (12 mM; specific radioactivity, 0.92 Ci/ mmol, 1.1 mCi) were dissolved in 100 μl of citrate buffer (50 mM, pH 4.5) containing 600 μg of Hex B (50 μM, 150 units) in a custom-made tapered quartz vial under safe light conditions. The solution was sonicated in an ice water bath for 5 s and deoxygenated by bubbling with argon for 2 min. After incubation at 37 °C for 15 min, the solution was irradiated for 20 min with UV light (λ\textsubscript{max} = 350 nm) in a Rayonet RPR 100 reactor (Southern New England Ultraviolet, Middletown, CT) equipped with 16 lamps (20 W each, RPR 3500 A). Inactivation was measured immediately after irradiation with 3 and 2 μl of the [3H]-1-ATB-GalNAc-labeled enzyme solution. The aliquots were diluted 1:20,000 with water containing 0.02% bovine serum albumin (w/v) and assayed for enzyme activity using MUG as a substrate. Under these conditions the concentration of photolyzed [3H]-1-ATB-GalNAc was lowered to approximately 1 μM. Protein concentration of additional aliquots (4 × 1 μl) were measured to determine the specific activity of the labeled enzyme. As control, a photolabeling experiment was conducted under the same conditions but in the absence of [3H]-1-ATB-GalNAc was performed and treated as above.

Photoaffinity Labeling of Hex B in Presence of δ-Lactone and Determination of Inactivation—193 μCi of [3H]-1-ATB-GalNAc (12 mM, 0.55 mCi) and 11 μCi of δ-lactone (1 mM) were dissolved in 50 μl of citrate buffer (50 mM, pH 4.5) containing 300 μg of Hex B (50 μM, 75 units) and treated as described above. Hex B activity after irradiation was determined as described above. Under these conditions the concentration of δ-lactone was lowered to approximately 0.05 μM.

Separation of β\textsubscript{α} and β\textsubscript{β}-Polypeptides—The entire labeled mixture obtained from the photoaffinity experiment of Hex B with [3H]-1-ATB-GalNAc was freeze dried and dissolved in 180 μl of Tris-HCl buffer (200 mM, pH 8.0, containing 6.6 mM guanidinium-HCl and 2.2 mM EDTA). The solution was incubated at 50 °C for 30 min. Disulfide bonds were reduced by the addition of 10 μl of dithiothreitol (1 mM) and incubation at 50 °C for 15 min. After cooling, 20 μl of iodoacetamide (12.5 mM) was added and allowed to react for 1 h at ambient temperature in the dark. The reaction was terminated by addition of 20 μl of β-mercaptoethanol. Subsequently, the entire mixture was loaded onto a Superdex 75 gel filtration column to remove photolysis byproducts, β-mercaptoethanol and iodoacetamide. Gel filtration was performed under denaturing conditions using 6 M guanidinium-HCl in phosphate buffer (20 mM, pH 5.8) as solvent. Aliquots of protein containing fractions were subjected to SDS-PAGE. Fractions containing both the β\textsubscript{α} and β\textsubscript{β}-polypeptide were pooled and subsequently separated by reverse phase HPLC as follows. HPLC grade water containing 0.1% trifluoroacetic acid was used as solvent A. Solvent B consisted of isopropanol containing 0.085% trifluoroacetic acid. Solvents were delivered using a LKB 2150 low pressure gradient system. Column temperature was maintained at 60 °C throughout the separation. A LiChrospher C\textsubscript{18} column (particle size, 10 μm; pore width, 100 Å; 4 × 250 mm) was equilibrated with 10% solvent B. The prepurified photolabeling mixture obtained from gel filtration was injected through a 2-ml loop and eluted with a gradient of 10–50% solvent B in 45 min and with 50% solvent B for 30 min at a flow rate of 1 ml/min. Following the absorption at 206 nm, fractions were cut manually in the range of 1.0–2.0 ml. Aliquots were assayed for radioactivity and subjected to SDS-PAGE. Fractions containing the β\textsubscript{α} and β\textsubscript{β}-polypeptide were pooled and freeze dried.

Tryptic Digest of β\textsubscript{α} and β\textsubscript{β}-Polypeptides—The separated and lyophilized β\textsubscript{α} and β\textsubscript{β}-polypeptides (5 nmol each) were dissolved in 100 μl of phosphate buffer (50 mM, pH 8.0, containing 6.6 mM guanidinium-HCl) and diluted to 1 ml with the same buffer containing 1 mM Ca\textsubscript{2+} instead of guanidinium-HCl. Modified trypsin (20 μg, final protease/
RESULTS

Enzyme Purification—Isolation of lysosomal Hex B from human liver resulted in a final preparation that was ~3000-fold enriched over the liver homogenate with an overall yield of ~10%. The specific activity was ~250 units/mg.

Covalent Binding of \(^{3}H\)-1-ATB-GalNAc to Hex B—Incubation of Hex B (50 \(\mu\)M) with \(^{3}H\)-1-ATB-GalNAc (12 \(\mu\)M; specific radioactivity, 0.92 Ci/mmol) and subsequent irradiation at 350 nm for 20 min resulted in a 15% reduction of the enzyme activity. The enzyme activity was not reduced in a control experiment in the absence of the photoaffinity label. However, in the simultaneous presence of the photoaffinity label (12 \(\mu\)M) and the \(\delta\)-lactone (1 \(\mu\)M) as a transition state analogue, enzyme activity was reduced by only 4% after irradiation indicating that the \(\delta\)-lactone prevented 75% of the covalent binding of the highly reactive carbene generated from \(^{3}H\)-1-ATB-GalNAc at the active sites of Hex B.

Subsequent size exclusion chromatography of the reduced and alkylated protein under denaturing conditions led to the isolation of two protein-containing peaks. The first major peak contained the \(\alpha\)-polypeptide and the second contained the \(\beta\)-polypeptide (Fig. 2). SDS-PAGE revealed that the first peak was enriched over the liver homogenate with an overall yield of ~10%. The specific activity was ~250 units/mg.

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Photoaffinity Labeling of Human Lysosomal Hex B

Fig. 3. Separation of $\beta_a$ and $\beta_b$-polypeptides of labeled Hex B by reverse phase HPLC. Photoaffinity labeling of Hex B with [3H]-1-ATB-GalNAc and separation of $\beta_a$ and $\beta_b$-chains by HPLC were conducted as described under "Experimental Procedures." Aliquots of each fraction were assayed for radioactivity. A, the chromatogram was obtained by HPLC separation of $\beta_a$- and $\beta_b$-polypeptides derived from labeled Hex B and the corresponding radioactivity profile. B, fractions designated a, b, c, and d in A were further analyzed by SDS-PAGE. Aliquots with equal amounts of radioactivity (about 0.1 $\mu$Ci) of fractions a–d were freeze-dried and separated by SDS-PAGE. The radioactive spots were identified by fluorography. Lane 1, [3H]-methylated molecular mass markers; lane 2, polypeptide mixture of $\beta_a$ and $\beta_b$ obtained by gel filtration of [3H]-1-ATB-GalNAc-labeled Hex B; lanes a–d, fractions designated a–d (see A).

An aliquot (~50 pmol) of this peptide fraction was directly subjected to automated sequence analysis. The effluent stream from the Edman degradation was split before it entered the phenylthiodyanton analyser, so that radioactivity of the effluent from each sequencing cycle could be correlated with analysis of PTH-derivatives. Automated Edman degradation of the labeled peptide yielded the sequence EISEVFPDQFIHLG (peptide I), corresponding to amino acid positions 339–359, within the preprosequence of human lysosomal $\beta$-hexosaminidase B. The sequence assignment was unambiguous, no secondary sequences were observed. However, contrary to our expectations, no significant burst of radioactivity and no severely reduced PTH-derivative signal were observed in any of the cycles during sequence analysis. All of the radioactivity was recovered in the sequencer waste. This result strongly indicated the presence of a labile ester bond between an acidic side chain of either a glutamic or aspartic acid and the photoaffinity label, which was then cleaved during sequencing under the conditions of Edman degradation.

For further analysis, the radiolabeled peptide (~50 pmol) was rechromatographed by narrow bore HPLC (Fig. 6) at pH 2.0 using the standard solvent system (0.1% trifluoroacetic acid in water versus 0.085% trifluoroacetic acid and 84% acetonitrile in water). As shown in Fig. 6A, rechromatography yielded one single peak comprising all of the radioactivity. However, its calculated specific radioactivity (0.066 Ci/mmol) was rather low and suggested a contamination by an unlabeled peptide. Therefore, the peak was rechromatographed in the solvent system of Serwe et al. (21) (0.2% hexafluoracetone in water, pH 7.0, versus 0.03% hexafluoracetone and 84% acetonitrile in water). As shown in Fig. 6B, the apparently homogenous peak (Fig. 6A) separated into three peaks designated peaks 1, 2, and 3, which were further analyzed by MALDI-TOF-MS and sequence analysis. The majority of radioactivity was found in peak 3. The specific radioactivity calculated for this peptide of 0.91 Ci/mmol is close to the specific radioactivity of [3H]-1-ATB-GalNAc indicating a 1:1 stoichiometry of labeling.

MALDI-TOF-MS gave a $(M+H)^+$ ion at m/z 2438.7 in the case of peak 1, which is close to the calculated mass of the peptide 1, E$^{339}$SEVFDPQFIHLG (359) with 2436.68. Peak 2 gave rise to a single molecular $(M+H)^+$ ion at m/z 3342.3, a value close to the calculated mass of an elongated peptide I (positions 339–366; 3338.7), plus iodoacetamide. In this minor cleavage product, the potential tryptic site at lysine 359 has apparently been skipped. In case of peak 3, a $(M+H)^+$ ion at m/z 2729.9 was found matching the calculated mass for...
was intermediate in retention between peaks 1 and 3. None of the peaks bore any residual radioactivity.

Both peptides were subjected to sequence analysis. The earlier eluting peptide was found to be peptide I (positions 339–359). The later eluting peptide had the same sequence, with the notable exception of cycle 17, where PTH-glutamine instead of PTH-glutamic acid was unambiguously identified. This result strongly suggests that [3H]-1-ATB-GalNAc was covalently attached with its aglycon to Glu-355 via an ester bond, which could be cleaved by aminolysis reverting one-half of the derivatized Glu-355 back to glutamic acid and converting the other half to glutamine.

**DISCUSSION**

Our attempts to probe the binding sites of hexosaminidases were initially based on the pyrrolidine aldehyde 2-acetamido-1,4-imino-1,2,4-trideoxy-o-galactitol, which was shown to act as a competitive inhibitor of enzyme activity on both the α- (Ki = ∼220 μM) and the β-subunit (Ki = ∼18 μM) of β-hexosaminidase A (28). In order to achieve covalent photoincorporation of this compound into hexosaminidases, we introduced a photolabile diazirine group via a butyl spacer at the nitrogen ring atom. Unexpectedly, incubation of β-hexosaminidase A with this photolabel even in concentrations up to 1 mM and subsequent irradiation did not result in any reduction of enzyme activity, although the inhibitory potential (Ki = ∼14 μM, β-subunit of β-hexosaminidase A) of the galactitol derivative was not affected by attachment of the photoaffinity label (data not shown). The ineffectiveness of certain carbohydrate-linked diazirines to react covalently with their receptors has been reported by Lehmann and Petry (29). However, 1-ATB-GalNAc, although showing only a moderate Ki value (∼3 mM) is able to reduce enzyme activity after binding and therefore was used as a photoaffinity label for purified Hex B.

Our results provide strong evidence that the amino acid sequence E339KSEVFPDPQIHGGDEVEFK359 is involved in the substrate binding site of human lysosomal β-hexosaminidase B. This conclusion is supported by the ability of δ-lactone to compete effectively for the active sites of the enzyme and to reduce labeling of the βα-polypeptide and peptide I with [3H]-1-ATB-GalNAc. The fact that photoincorporation of the label was not totally prevented was somewhat surprising, because addition of 1 mM δ-lactone into the labeling experiment should completely protect the active sites of Hex B from covalent reaction with [3H]-1-ATB-GalNAc. On the other hand, it is known that the δ-lactone is an inactive or labile and in equilibrium with 2-acetamido-2-deoxy-o-galactone-1,4-lactone and 2-acetamido-2-deoxy-o-glucuronolactone (30). Therefore we compared the inhibitory potential of a freshly prepared aqueous solution of δ-lactone (1 mM) with the activity of a δ-lactone solution (1 mM) treated under the labeling conditions. It turned out that a freshly prepared aqueous solution of δ-lactone showed a Ki value (∼25 mM) that was about 20 times lower than the solution of δ-lactone that ran through the whole labeling procedure (Ki = ∼500 mM) (data not shown). Referring this result to the competition experiment, it is likely that the initially applied amount of δ-lactone (1 mM) is severely reduced during the labeling experiment, so that the δ-lactone concentration appears to be in the same order of magnitude as the enzyme concentration (50 μM) or even below the active site concentration (100 μM). Under these conditions, complete protection of labeling by the δ-lactone cannot be expected anymore.

Laser desorption mass spectrometry of the predominantly labeled peptide identified peptide I with a single affinity ligand attached. During the isolation and purification procedures of peptide I, it was noticed that the labeled peptide was treated with strongly acidic or basic solutions.

**Fig. 5.** Separation of the tryptic digest of the βα-polypeptide after labeling with [3H]-1-ATB-GalNAc by reverse phase HPLC. A, HPLC separation of peptides derived from the labeled βα-polypeptide. The arrow indicates the labeled peptide that was further purified by narrow bore HPLC and subjected to sequence analysis. Photoaffinity labeling of Hex B with [3H]-1-ATB-GalNAc, separation of βα- and ββ-chains, digestion of labeled βα-polypeptide with trypsin, and HPLC analysis were conducted as described under "Experimental Procedures." B, the corresponding radioactivity profile to A as obtained by liquid scintillation counting. C, control to B radioactivity profile obtained after labeling of Hex B in the presence of the competitive inhibitor δ-lactone (1 mM) to demonstrate the specificity of the reaction by reduced label incorporation into the peak at 52 min. Experimental details are provided under "Experimental Procedures."
performed the entire photoaffinity labeling experiment with
radiation. Exactly the same results were obtained when we
conversion of Glu-355 into Gln-355, as shown by Edman deg-
cleavage of the label from the peptide and simultaneously,
ammonium hydroxide (22), which resulted in quantitative
Therefore, we treated the labeled peptide I with 25% aqueous
be hydrolyzed under strongly acidic or basic conditions (31).
ticonductor nique have been recently reviewed by Withers and co-workers
liver (data not shown).
The mechanisms of enzymatic glycoside hydrolysis and the
approaches to identify active site residues by labeling tech-
niques have been recently reviewed by Withers and co-workers
(32, 33). In case of retaining glycosidases, such as hexosami-
idases (34), it is generally known that hydrolysis of a glycoside
bond is accomplished by a pair of acidic side chains, which
participate simultaneously in this reaction. The carboxyl group
of one of these side chains functions as a general acid and base,
whereas the other one acts as a nucleophile and a leaving
group. In this context, it is interesting that in our experiments
only one acidic amino acid is exclusively labeled by an active
site-directed affinity ligand. The labeling is highly specific,
because peptide I is the only peptide carrying significant
amounts of radioactivity, and furthermore the labeling reaction
can be suppressed to a significant extent by the addition of a
competitive but nonreactive inhibitor. Moreover, the reaction
happens to the side chain of Glu-355 exclusively and extends
to neither Asp-354 nor Glu-357, making it unlikely that the car-
bene just picks out the best nucleophile around. On the other
hand, the photoreactive site within the butyl residue is rather
remote from the bound sugar and furthermore possesses an
inherent flexibility, making it unlikely to assign labeled Glu-
355 to one of the amino acids involved in catalysis. With the
help of naturally occurring mutations of the B1 variant (re-
viewed in Ref. 35), Brown and Mahuran (36) reported that Arg-178 of the α-subunit (corresponding to Arg-211 on the
β-subunit) could be implicated in the active site of human
hexosaminidase. Although the data strongly suggest that
these residues are important for the activity of hexosamin-
dase, the assignment that they participate in the cleavage of
the glycosidic bond is not compelling. Moreover, as shown by
molecular modeling, mutations of these basic amino acids re-
sult in a severe disruption of the three-dimensional confor-
amation of the subunits (35) and therefore do not prove that α-sub-
unit Arg-178 or β-subunit Arg-211 are involved catalytically in
the active site. Although our results of photoaffinity labeling of
the binding site of Hex B with 1-ATB-GalNAc focus attention
on Glu-355, full understanding of the functional aspects of this
amino acid will undoubtedly await elucidation of the tertiary
structure of Hex B.

Similarly, identification of the modified amino acid within the
sequence of peptide I by Edman degradation failed, presumably
because the protocols used for automated sequencing include
treatment with 25% aqueous trifluoroacetic acid for 20 min at
64 °C. These observations led to the assumption that the pho-
tolabel was bound to the peptide via an ester bond that would
bene just picks out the best nucleophile around. On the other
hand, the photoreactive site within the butyl residue is rather
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unit Arg-178 or β-subunit Arg-211 are involved catalytically in
the active site. Although our results of photoaffinity labeling of
the binding site of Hex B with 1-ATB-GalNAc focus attention
on Glu-355, full understanding of the functional aspects of this
amino acid will undoubtedly await elucidation of the tertiary
structure of Hex B.

Because this is, to our knowledge, the first identification of a
specific sequence at the binding site of the β-subunits from
human hexosaminidase B, we were interested in the extent to
which this sequence is conserved among hexosaminidases from
other species and human α-chain in particular. Alignment of
amino acid sequences for hexosaminidases from various species
revealed that Glu-355 is highly conserved between the α-sub-
unit of human β-hexosaminidase A, the α- and β-subunit of
mouse, and the α-subunit of Dictostelium discoideum (Fig. 7).
Moreover, there are several highly conserved amino acids in
the vicinity of Glu-355, being potential targets for future
studies using site-directed mutagenesis to probe the binding
site in hexosaminidases. Interestingly, Glu-355 is located near
Cys-360, which is bridged with Cys-309, connecting the βα and
the βα-polypeptides in Hex B. These cysteine residues and also
the size of the loop they enclose are conserved in all known
hexosaminidase subunit sequences (37).

It is clear from previous attempts to locate the active sites of
hexosaminidases that there are several regions of homology among them. One was found using the predicted amino acid sequence of the bacterial β-N-acetylhexosaminidase gene from Vibrio vulnificus. Sequence alignments identified a region of 19 amino acids largely conserved among the central portion of the amino acid sequence among known hexosaminidases but not including Glu-355 (38). Although regions of homology that have been identified by sequence alignments may prove to be important in the structure of the active sites of hexosaminidases, our results with photoaffinity labeling and peptide sequencing provide the first chemical evidence identifying a specific amino acid at the binding site of Hex B.

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