Identification of an Active Acidic Residue in the Catalytic Site of β-Hexosaminidase†

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Received January 31, 1996; Revised Manuscript Received April 5, 1996⊗

ABSTRACT: Human β-hexosaminidases A and B (EC 3.2.1.52) are dimeric lysosomal glycosidases composed of evolutionarily related α and/or β subunits. Both isozymes hydrolyze terminal β-linked GalNAc or GlcNAc residues from numerous artificial and natural substrates; however, *in vivo* G_{M2} ganglioside is a substrate for only the heterodimeric A isozyme. Thus, mutations in either gene encoding its α or β subunits can result in G_{M2} ganglioside storage and Tay-Sachs or Sandhoff disease, respectively. All glycosyl hydrolases are believed to have one or more acidic residues in their catalytic site. We demonstrate that incubation of hexosaminidase with a chemical modifier specific for carboxyl side chains produces a time-dependent loss of activity, and that this effect can be blocked by the inclusion of a strong competitive inhibitor in the reaction mix. We hypothesized that the catalytic acid residue(s) should be located in a region of overall homology and be invariant within the aligned deduced primary sequences of the human α and β subunits, as well as hexosaminidases from other species, including bacteria. Such a region is encoded by exons 5−6 of the *HEXA* and *HEXB* genes. This region includes βArg_{211} (invariant in 15 sequences), which we have previously shown to be an active residue. This region also contains two invariant and one conserved acidic residues. A fourth acidic residue, $Asp^{\alpha 258,\beta 290}$, in exon 7 was also investigated because of its association with the B1 variant of Tay-Sachs disease. Conservative substitutions were made at each candidate residue by *in vitro* mutagenesis of a βcDNA, followed by cellular expression. Of these, only the $\beta Asp^{196}Asn$ substitution decreased the k_{cat} (350−910-fold) without any noticeable effect on the K_m. Mutagenesis of either βAsp^{240} or βAsp^{290} to Asn decreased k_{cat} by 10- or 1.4-fold but also raised the K_m of the enzyme 11- or 3- fold, respectively. The above results strongly suggest that βAsp^{196} is a catalytic acid residue in β-hexosaminidase.

β-Hexosaminidase (Hex)[1] is a lysosomal hydrolase that cleaves terminal β-*N*-acetylhexosamines from oligosaccharides, glycolipids, glycoproteins, and glycosaminoglycans. The human enzyme occurs as two major isozymes, Hex A ($\alpha\beta$) and Hex B ($\beta\beta$). Both of the Hex isozymes are able to hydrolyze many of the same substrates, but only Hex A, in combination with a specific activator protein, can hydrolyze G_{M2} ganglioside *in vivo*. Mutations that lead to the absence or malfunction of the enzyme or the activator protein result in severe inherited neurodegenerative diseases, known as the G_{M2} gangliosidoses. One such disorder, Tay-Sachs disease, results from mutations in the *HEXA* gene encoding the α subunit of Hex A. Another, Sandhoff disease, results from mutations in the *HEXB* gene affecting the common β subunit [reviewed in Gravel et al. (1995)].

Both the α and β subunits are synthesized in the ER as larger precursor polypeptides and are processed to their mature forms in the lysosome [reviewed in Gravel et al. (1995)]. Exit from the ER to the Golgi requires that all proteins, whether secretory, plasma membrane, or lysosomal, acquire their near native conformation. Acquiring a native confirmation includes, for some proteins like Hex, the formation of dimers (Edgington, 1992; Hurtley & Helenius, 1989; Pelham, 1989; Proia et al., 1984). Many point mutation that cause Hex A deficiencies result from the retention of the mutant protein in the ER [reviewed in Mahuran (1991)]. An interesting exception to these "folding" mutations are those associated with the B1 variant of Tay-Sachs disease (Fernandes et al., 1992; Kytzia et al., 1983; Ohno & Suzuki, 1988; Tanaka et al., 1988), $\alpha Arg^{178}His$ and $\alpha Asp^{258}His$. The B1 variant has an unusual phenotype. Patient samples contain both the A and B isozymes; however, the Hex A lacks activity toward MUGS and G_{M2} ganglioside. Thus these mutations are thought to affect residues at or near the α-active site.

Comparison of the deduced primary structures of the human α and β subunits demonstrates an almost 60% homology, suggesting a common evolutionary origin (Korneluk et al., 1986). The hypothesis that the two genes evolved from a common ancestral gene is further substantiated by the striking similarities in the number and placement of intron/exon junctions within the *HEXA* and *HEXB* genes (Neote et al., 1988; Proia, 1988). Thus, regions in these aligned sequences that have been well conserved are likely

† This work was funded through a grant to D.M. from the Medical Research Council of Canada.

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⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1996.

[1] Abbreviations: MUG, 4-methylumbelliferyl-β-*N*-acetylglucosamine; MUGS, methylumbelliferyl-β-*N*-acetylglucosamine-6-sulfate; MU, methylumbelliferone; G_{M2} ganglioside, GalNAcβ(1−4)[NeuAcα(2−3)]-Galβ(1−4)Glc-ceramide; ER, endoplasmic reticulum; Hex, β-hexosaminidase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DSP, dithiobis(succinimidylpropionate); EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

S0006-2960(96)00246-2 CCC: $12.00

Table 1: Origin of Hex-Related Sequences Identified in a Local Search for Homologies with Various Sections of Coding Sequence from the Human *HEXB* Gene (See Table 4)

abbreviations[a]	species	accession no.	reference
HumA	human Hex α-subunit	P06865[b]	(Korneluk et al., 1986)
MuA	mouse Hex α-subunit	P29416[b]	(Beccari et al., 1992)
HumB	human Hex *β*-subunit	M13519[b]	(Korneluk et al., 1986)
MuB	mouse Hex *β*-subunit	P20060[b]	(Bapat et al., 1988)
CatB	feline Hex *β*-subunit	S70340[c]	(Muldoon et al., 1994)
Boar	pig Hex epididymal protein (*β*-subunit)	X92379[c]	Syntin[e]
DicDi	*Dictyostelium discoideum* Hex	P13723[b]	(Graham et al., 1988)
EntAmo	*Entamoeba histolytica* Hex	U09735[c]	(Beanan & Bailey, 1995)
Vib-para	*Vibrio parahaemolyticus* cytoplasmic chitobiase	U24658[c]	Wu[f]
Porphyr	*Porphyromonas gingivalis* W83 outer-membrane-associated lipoprotein Hex	X78979[c]	(Lovatt & Roberts, 1994)
Can-alb	*Candida albicans* (yeast) Hex	P43077[b]	(Cannon et al., 1994)
SilkW	*Bombyx mori* (silkworm), chitooligosaccharidolytic NAG[d]	JC2539[c]	(Nagamatsu et al., 1995)
Alter	*Alteromonas sp.* (strain 0−7, marine bacterium) NAG	D29665[c]	(Tsujibo et al., 1994)
Vib-vul	*Vibrio vulnificus* (scotobacteria) chitooligosaccharidolytic NAG	Q04786[b]	(Somerville & Colwell, 1993)
Vib-har	*Vibrio harveyi* (scotobacteria) outer membrane, lipoprotein; chitooligosaccharidolytic NAG	P13670[b]	(Soto-Gil & Zyskind, 1989)

[a] Used in Figure 2 and Table 4. [b] SWISS-PROT Protein Sequence Database (32.0, 11/95). [c] GenBank (92.0, 12/18/95). [d] *β*-*N*-Acetylglucosaminidase. [e] P. Syntin, N. Okamura, F. Guillou, F. Dacheux, and J. L. Dacheux, (unpublished results. [f] M. H. Wu, and R. A. Laine, (unpublished results).

important for proper folding, substrate binding, or catalysis. This hypothesis is now generally accepted for Hex and other glycosyl hydrolases which have been grouped into families based on sequence homology; Hex has been placed in family 20 (Henrissat, 1991; Henrissat & Bairoch, 1993).

In a previous report, we analyzed the biochemical consequences of the B1-substitution in the α subunit, αArg^{178}His, by *in vitro* mutagenesis of the homologous codon in the *β*-subunit, *β*Arg^{211}His. In our study with this *β*-analog, we noted small changes in the stability and the rate of processing of the mutant protein (Brown et al., 1989) which were totally eliminated when a more conservative substitution, *β*Arg^{211}Lys, was introduced. Whereas this mutant Hex B retained a normal K_m for MUG, its V_{max} was reduced by greater than 400-fold and its pH optimum shifted. From these and other data, we concluded that αArg^{178} and *β*Arg^{211} are active site residues, i.e., part of the catalytic sites, in Hex (Brown & Mahuran, 1991; Hou et al., 1996). The other substitution linked to the B1 variant, αAsp^{258}His, has not been studied by any form of *in vitro* mutagenesis and cellular expression (Fernandes et al., 1992).

In this report we demonstrate the presence of an active acidic group and examine four candidate residues by *in vitro* mutagenesis. Within 15 aligned family 20 sequences, there are only two invariant and one conserved acidic amino acid residues. These are *β*Asp^{196} (invariant), *β*Asp^{208} (invariant), and *β*Asp^{240} (conserved, i.e., identical in 14/15, Glu in the remaining). Site-directed mutagenesis was performed on these acidic residues, as well as on *β*Asp^{290} (identical in 10/15), because its α homologue is associated with the B1 variant form of Tay-Sachs, and on *β*Glu^{253} (identical in 8/15) to explore the effects of a conservative substitution on a less conserved acidic residue. The effects on enzymatic activity, intracellular transport, kinetics, and dimer formation of Hex B in transfected COS-1 or CHO cells were analyzed.

MATERIALS AND METHODS

Protein Homology Alignments. Hex-related sequences were identified using a local search procedure [BLASTP/nr protein, with Entrez & SRS links (NCBI/BCM)], from the Human Genome Center, Baylor College of Medicine, available through the World Wide Web (http://kiwi.imgen.bcm.tmc.edu:8088/search-launcher/launcher.html). Amino acids encoded by exons 1−4 (excluding the signal peptide sequence), 4−7, 8−10, and 11−14 of the human *HEXB* gene were independently searched for homologous sequences. The entire coding sequences of 15 Hex-like enzymes (including human Hex B) identified by these searches (Table 1) were then aligned by a global protein alignment program from GeneWorks (IntelliGenetics, Inc.).

Site-Directed Mutagenesis and Vector Constructions. Cloning procedures were as described by Sambrook et al. (1989). All mutant cDNA inserts were fully sequenced by dideoxy sequencing (Sanger et al., 1977).

The cDNA construct pHexB43 encoding for the *β*-subunit of Hex was digested with *Eco*R1, and a 496-base pair fragment generated was subcloned into a Bluescript vector [pBs(+)] (Stratagene). A single-stranded DNA template was generated from the Bluescript construct upon superinfection with helper phage M13K07. The mutagenesis was subsequently performed utilizing Amersham Corporation protocols based on the original method described by Taylor et al. (1985). The mutant oligos used were designated oligo 1 (Asp^{196}Asn), oligo 3 (Asp^{240}Asn), and oligo 5 (Glu^{253}Gln) (Table 2). The mutated fragments were subsequently subcloned back into pHexB43 for transient COS cell expression studies.

Point mutations encoding Asp^{208}Asn and Asp^{290}Asn were constructed using two alternate procedures. For the Asp^{208}Asn substitution, pSVL-*β* was mutated utilizing the Transformer Mutagenesis Kit (Clonetech Lab. Inc.) and oligo 2 (Table 2). The second mutation, Asp^{290}Asn, was constructed utilizing a variation of the PCR fusion technique (Yon & Fried, 1989). The principle of PCR-mediated gene fusion involved the utilization of three different primers within the two major steps. In step 1, 35 cycles of amplification were performed using a "linking" oligo/primer [oligo 4 (Asp^{290}Asn) Table 2], which for our purpose was complementary to only the *β*cDNA, i.e., we were not using it to make a fusion protein, and contained the appropriate nucleotide change to encode the substitution, along with the 3′ end primer (oligo 6, complementary to the 3′ end sequence of the Hex B cDNA). The product (termed "intermediate") containing the point mutation of interest then acts as a "primer" in conjunction with the 5′ end primer (oligo 7, complementary to the 5′ end sequence of the *β*cDNA) to yield the final product of the reaction in step 2 of the process (Table 2).

Table 2: Oligonucleotides Utilized to Mutate βcDNA[a]

number	oligonucleotide	change	mutation induced
1	5′-TCCACCATTATTAACTCTCCAAGGGTTT-3′	GAT → AAC	$Asp_{196}Asn$
2	5′-GGAATTTTGATTAATACATCCAGA-3′	GAT → AAT	$Asp_{208}Asn$
3	5′-GCACATAGTTAACGACCAGTCTTT-3′	GAT → AAC	$Asp_{240}Asn$
4	5′-ACGAGGAATTCGAGTCCTGCCAGAATTTAATACCCCTGGG-3′	GAT → AAT	$Asp_{290}Asn$
5	5′-ATCACTTTTCCTCAGTTAAGCAATAAA-3′	GAG → CAG	$Glu_{253}Gln$
6	5′-GCGGCCGCCATTTTTTACATGTTCTCATGGTTACA-3′	3′-primer	none
7	5′-GCGGCCGCATGCTGCTGG-CGGTGCTGTTGGCGACACTG-3′	5′-primer	none

[a] The nucleotide changes are underlined, and the changes consequently made in the deduced amino acid sequence of the β-subunit are also shown.

The mutant cDNA inserts were subcloned into pEFNEO for permanent expression in CHO cells.

Cell Culture. COS-1 cells were obtained from the American Type Culture Collection, Rockville, MD. They were maintained and propagated in α-minimal essential media supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 μg/mL penicillin, at 37 °C in 5% CO_2. Chinese hamster ovary tumor cells (CHO) were maintained and propagated in the same manner.

Transient DNA Transfection. Two different methodologies were employed to transfect DNA into COS-1 cells, calcium phosphate and liposome-mediated transfection. With the former, 10 μg of pHexB43 (containing the wild type or mutant prepro-β-cDNA insert) was transfected into COS-1 cells (Chen & Okayama, 1987). As well, COS-1 cells were cotransfected with 10 μg of pBLCAT2, a pUC18 derived plasmid containing the coding region of the bacterial CAT gene, used to evaluate transfection efficiency from one cell culture dish to the next (Neumann et al., 1987). In the case of the Lipofectin-mediated transfections, 8 μg of pHexB43 and 3 μg of pBLCAT2 were transfected into COS-1 cells essentially according to GIBCO-BRL procedure (Felgner et al., 1987). Transient expression was carried out for 72 h, and cells were harvested as described (Neote et al., 1990).

Permanent DNA Transfection. Ten micrograms each of the pEFNEO vector containing the wild type or mutant Hex cDNA inserts were transfected into CHO cells using Lipofectin. The transfection procedure was as suggested by GIBCO-BRL. Antibiotics, G-418/neomycin, selection was started 48−72 h after the transfection. The media of surviving clones were analyzed for Hex activity. In the case of cells expressing the wild type insert, this method allowed for the establishment of a clonal line producing 100-fold more Hex activity than the average surviving clone. With cells transfected with mutant βcDNAs producing little activity, this method could not be used efficiently. Thus levels of β-protein had to be quantitated in order to compare the specific activity of mutant and normal Hex B enzymes (see below).

Hexosaminidase Enzyme Assays. Human Hex activity (MUG) in transfected cells' lysates or media was determined using the human Hex-specific solid-state immunoprecipitation (Brown et al., 1989). CHO cells were used in the latter part of this study because our antiserum that could distinguish between COS and human Hex was exhausted. Several other antisera could be made human specific by absorbing them with CHO cell lysate bound to Concanavalin A Sepharose. Unfortunately, absorption of these antisera with COS cell lysate resulted in a loss of cross-reaction to both human and COS Hex (data not shown).

Inactivation of β-Hexosaminidase A and B by 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). Hex A and B were purified from human placenta by the method of Mahuran and Lowden (1980). Fifteen microliters of Hex A (100 ng) was incubated with 100 mM EDC in 85 μL of 10 mM sodium phosphate buffer, pH 6.0, containing human serum albumin at a final concentration of 1 mg/mL. Aliquots, 10 μl, were removed at time intervals (every 0.5 h for a total of 4 h) and diluted into 90 μl of cold McIlvaine (citrate/phosphate) buffer, pH 4.1, containing 0.3% human serum albumin for assay (Brown & Mahuran, 1991; Tommasini et al., 1985b), with the α-specific (Hex A) artificial substrate MUGS and the common (Hex A and B) MUG substrate.

Inhibition of EDC Inactivation of Hexosaminidase A by 2-Acetamido-2-deoxy-D-Galactonolactone. Hex A was incubated for 2 h with 50 mM 2-acetamido-2-deoxy-D-galactonolactone in 10 mM sodium phosphate buffer, pH 6.0, containing human serum albumin at a final concentration of 1 mg/mL. Thereafter, EDC was added to the reaction (final concentration of 100 mM). Following a 2 h incubation at room temperature, the EDC reaction was quenched by the addition of ammonium acetate (100 mM). Samples were then dialyzed overnight against 0.5 M Tris-HCl, pH 8, in order to remove the lactone. Subsequently the sample was dialyzed for 2 h against McIlvaine's buffer (pH 4.1) prior to being assayed for MUG and MUGS activity.

Determination of the Apparent K_m and V_{max}. The apparent K_m and V_{max} for the wild type and the mutant Hex B proteins were determined using MUG substrate concentrations ranging from 0.4 to 4.0 mM. The solid-state human Hex-specific assay was used (see above). Values obtained were reduced by the units of activity found in an identical immunoprecipitated sample of mock transfected cell lysate. The data were analyzed using Kaleidagraph, a computerized nonlinear least-squares curve fitting program for the Macintosh, based on the Michaelis−Menten equation, $V_i = V_{max}[S]/(K_m + [S])$. Thus, accurate standard errors could be calculated for K_m and V_{max} (Tommasini et al., 1985a).

Chemical Cross-Linking with Dithiobis(succinimidylpropionate) (DSP). Transfected COS-1 (or CHO) cell lysates were prepared as described previously (Brown et al., 1989) with the exception that the lysis buffer utilized was comprised of the following: 50 mM sodium phosphate, pH 7.0, 150 mM NaCl, 10 mM $MgCl_2$, 0.05% Triton X-100, 5 mM β-glycerophosphate, and 0.05% bovine serum albumin. Lysates were subsequently adjusted to 0.6 mM DSP using a 6 mM stock solution in dimethyl sulfoxide. Controls received dimethyl sulfoxide only. After a 30 min incubation at 37 °C, the reaction was quenched by the addition of Tris-HCl, pH 7.5, to bring it to 100 mM. Nonreduced lysates

were subsequently separated by SDS−PAGE for Western blot analysis.

Western Blot. COS-1 lysates were analyzed using SDS−PAGE. Lowry assays were done in order to calculate the total protein present. Western blots were developed by the peroxidase antiperoxidase procedure (Jackson Immunological) using a donkey anti-rabbit IgG as the bridging antibody and normal donkey serum as the blocking agent, as previously reported (Brown et al., 1989).

Permanently transfected and control (-ve) CHO lysates were also analyzed using SDS−PAGE and Western blotting as above. Additionally, the peroxidase-visualized protein bands, corresponding to mature β chain, were quantitated against the wild type protein by densitometry (Shea, 1994). As well, in some cases lysate proteins were transferred to ECL H-Bond Nitrocellulose Membrane. These Western Blots were subsequently visualized by the Chemiluminescence (ECL)/Hyperfilm-ECL (Amersham) procedure (Xie et al., 1992). Whereas this latter procedure is far more sensitive that the peroxidase stain, it produces an exponential rather than a linear response with increasing amounts of Hex protein when scanned by densitometry (data not shown).

RESULTS

Initially, experiments were undertaken to investigate the possible role of carboxyl groups as active residues in Hex through the treatment of the purified placental isozymes with EDC. Water-soluble carbodiimides have been shown to specifically interact with protonated carboxyl groups (Hoare & Koshland, 1967). One such compound, EDC, has been used to modify carboxyl groups at the active site of lysozyme and a number of other enzymes (Carraway & Koshland, 1972; Chan et al., 1988). Incubation of purified placental Hex A at pH6 with EDC results in a rapid, time-dependent loss of enzymatic activity associated with both the α- (as assessed by MUGS, Figure 1, panel A) and β- (as assessed by MUG, Figure 1, panel B) subunits of the heterodimer. Treatment of Hex B with 100 mM EDC also resulted in progressive inactivation of the enzyme as assayed with the MUG substrate (Figure 1, panel B). Inactivation of both enzymes was also found to be EDC concentration dependent (data not shown). These data suggest that acidic amino acid residues are involved in the active sites of both subunits.

In order to confirm that the above inactivation of Hex A and B by EDC was not caused by an indirect effect on protein conformation, the reaction was repeated in the presence of a strong competitive inhibitor, 2-acetamido-2-deoxy-D-galactonolactone (Kanfer & Spielvogel, 1973; Pocsi et al., 1990; Sandhoff et al., 1977). When this competitive inhibitor was allowed to interact with the purified placental Hex A prior to its EDC modification, a significant protection against enzymatic inactivation was observed (Table 3). These data are consistent with the presence of an "active" carboxyl group, i.e., protonated at pH 6 due to the microenvironment of the active site, present in both the α and β subunits of Hex A.

The above data, suggesting the presence of protonated, active acid residues in both the α and β subunits of Hex, caused us to search the deduced primary structure of the β subunit for candidate sites to be analyzed by *in vitro* mutagenesis and COS or CHO cell expression. Although substrate binding sites may vary, the domains containing catalytic residues of related glycosyl hydrolases have been

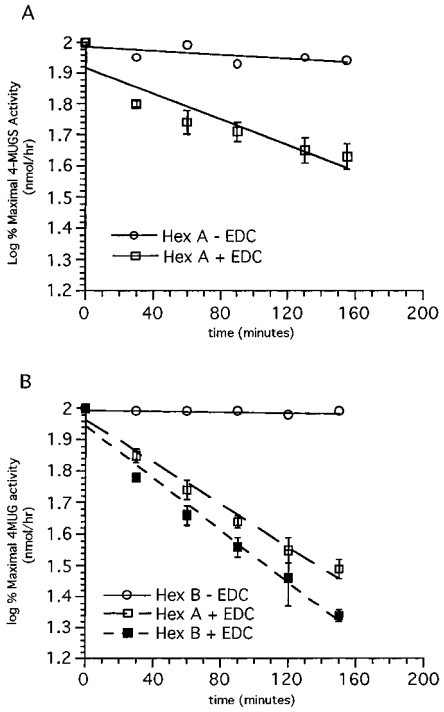

FIGURE 1: Inactivation of the α- and β-catalytic sites of Hex by EDC. The enzyme was incubated at 25 °C with 100 mM EDC. Samples were withdrawn at the indicated times and assayed immediately. (Panel A) Inactivation of the α-active site of Hex A as assessed with MUGS; (panel B) inactivation of the α- and/or β-active sites of Hex A and Hex B as assessed with MUG. The log (% activity remaining) is plotted against incubation time. Data points are shown fitted to a linear equation by least-squares analysis. Error bars are standard deviations based on results from three separate experiments.

Table 3: Inhibition of EDC Inactivation of Hex A by 2-Acetamido-2-deoxy-D-galactonolactone[a]

sample	50 mm lactone	dialysis pH 8.5	EDC	%MUG activity	%MUG activity
Hex A	−	−	−	100	100
Hex A	+	−	−	4 ± 1	6 ± 2
Hex A	+	+	−	90 ± 3	88 ± 8
Hex A	−	−	+	27 ± 4	21 ± 5
Hex A	+	+	+	83 ± 5	85 ± 6

[a] "−", not treated with; "+", treated with.

shown to be conserved throughout evolution. Additionally, the acid residues involved in catalysis remain invariant in aligned sequences (Damude et al., 1995; Henrissat, 1991). Thus four regions of human Hex B were searched for local homologies with other family 20 enzymes that hydrolyze β-*N*-acetylglucosamine and/or galactosamine-containing substrates. The domain comprising exons 5−7 was found to be the most conserved coding region in the human *HEXB* gene (Table 4). These results were confirmed by aligning all 15 of the primary structures from the enzymes identified in the local searches, using a global protein alignment program. The only area containing invariant residues was between βIle^{194} and βHis^{237} (Figure 2). This region contained two invariant acidic residues, βAsp^{196} and βAsp^{208}. A residue slightly outside this region, βAsp^{240}, was identical in all but one sequence where it aligned with a Glu. These three residues were selected for further study along with βGlu^{253}, a less well conserved residue used as a control, and βAsp^{290} which aligns with αAsp^{258}. When substituted by His in the α subunit, i.e., αAsp^{258}His, the B1 variant form of Tay-Sachs disease results (Fernandes et al., 1992).

Table 4: Search for Local Homologies in the Deduced Coding Sequences from Groups of Exons from the Human *HEXB* Gene

abbreviation[a]	accession no.	exons 1−4 (β: 43−187)[b,c]	exons 5−7 (β: 188−299)[b]	exons 8−10 (β: 300−414)[b]	exons 11−14 (β: 415−556)[b]
HumA	P06865	6.4×10^{-29} [d]	1.3×10^{-53}	5.8×10^{-54}	1.4×10^{-56}
MuA	P29416	2.7×10^{-31}	1.9×10^{-54}	1.0×10^{-48}	2.5×10^{-55}
HumB	M13519	3.0×10^{-103} [e]	3.7×10^{-77} [e]	2.0×10^{-80} [e]	1.1×10^{-103} [e]
MuB	P20060	1.7×10^{-70}	9.9×10^{-70}	2.6×10^{-60}	7.1×10^{-76}
CatB	S70340	8.1×10^{-62}	2.0×10^{-70}	1.1×10^{-61}	3.7×10^{-77}
Boar	X92379	8.2×10^{-56}	1.7×10^{-65}	8.4×10^{-43}	5.0×10^{-77}
DicDi	P13723	3.9×10^{-05}	8.9×10^{-36}	4.2×10^{-10}	2.4×10^{-23}
EntAmo	U09735	0.997	1.1×10^{-23}	6.0×10^{-10}	8.3×10^{-15}
Vib-para	U24658	1.0[f]	2.2×10^{-16}	0.026	0.95
Porphyr	X78979	1.0	9.1×10^{-21}	3.7×10^{-05}	1.0
Can-alb	P4307	0.010	2.9×10^{-31}	2.2×10^{-04}	1.2×10^{-08}
SilkW	JC253	0.96	8.9×10^{-33}	8.1×10^{-08}	6.5×10^{-10}
Alter	D29665	1.0	3.8×10^{-18}	1.0	1.0
Vib-vul	Q04786	1.0	1.5×10^{-21}	1.0	1.0
Vib-har	P13670	1.0	1.9×10^{-19}	1.0	1.0

[a] For an explanation of these abbreviations, see Table 1. [b] Residues in the search sequence, the numbering system is from the human β cDNA. [c] The N-terminal signal sequence, β 1−42, was not included in the search. [d] The *P* value (probability in the range 0−1) of observing such a match in a random sequence from the data base; a BLASTP 1.4.6MP [13-Jun-94] [Build 13:58:36 Sep 22 1994] local search (Altschul et al., 1990). [e] *P* value obtained from a perfect match. [f] No homologous sequences from this enzyme were found in the search of the indicated human Hex B sequence and the *P* value is thus 1.0.

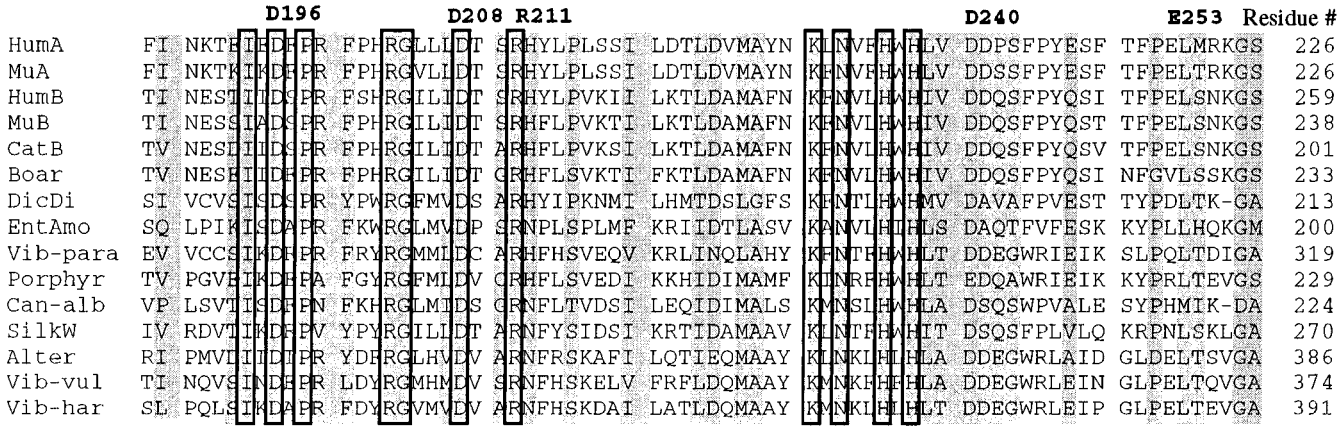

FIGURE 2: Section of the total aligned deduced amino acid sequences of Hex from 15 species (Table 1). This region shows the greatest degree of homology as determined by both local (Table 4) and global alignment programs. It also contains all the invariant residues found within the alignments. Boxed residues are invariant, whereas shaded residues have some conservative substitutions. Numbered (based on the human β-sequence) acidic residues in bold at the top of the figure are those chosen for mutational analyses. R^{211} (Arg^{211}) is the β residue that aligns with αR^{178} which is associated with the B1 variant of Tay-Sachs disease. We have previously presented strong evidence that this invariant amino acid is an active residue in the catalytic site of Hex.

The effects of conservative, neutral substitutions for each of the above acidic residues on the levels of mature β-chain protein and Hex B activity produced in transfected cells were determined. Mature, lysosomal β-protein was detected in Western blots from all (Figures 3−5) but the CHO cell line transfected with the construct encoding the $Asp^{208}Asn$ substitution (Figure 5). In this line the only immunoreacting band had a M_r corresponding to that of the β-precursor chain, a form found primarily in the ER (Hasilik & Neufeld, 1980; Proia et al., 1984) (Figure 5). These data indicate that the acidic side chain of Asp^{208} is very important in the initial folding and/or stability of the proβ-chain and is thus unlikely to be involved in substrate catalysis. A small increase in the proβ form was also found in CHO cells expressing the $Asp^{290}Asn$ mutation (Figure 5). This suggests that the substitution has affected the rate of protein folding and/or dimerization. After small adjustments for transfection efficiency, using cat activities (data not shown), the mature β-protein levels in the COS cells expressing three of the mutant Hex Bs were nearly identical to those expressing the wild type protein (Table 5, column 3). This made specific activity calculations (see below) straightforward. However,

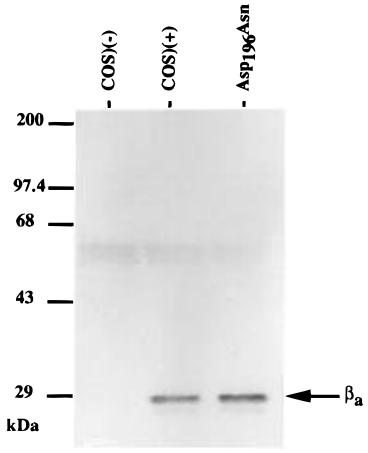

FIGURE 3: Western blot of lysate from normal COS cells (COS (−)) and those transiently transfected with either wild type βcDNA (COS (+)) or mutant βcDNA encoding a $Asp^{196}Asn$ substitution. Samples of each lysate contain equal amounts of reduced protein (50 μg).

expression levels in cloned, permanently transfected CHO cells varied greatly. The clone expressing the wild type Hex

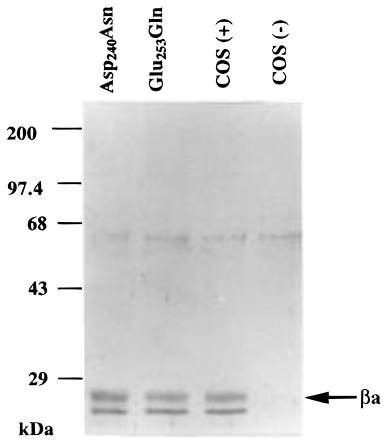

FIGURE 4: Western blot of lysate from normal COS cells (COS (−)) and those transiently transfected with either wild type βcDNA (COS (+)) or mutant βcDNA. The mutations encode either a Glu^{253}Gln or a Asp^{240}Asn substitution. Samples of each lysate contain equal amounts of reduced protein (50 μg).

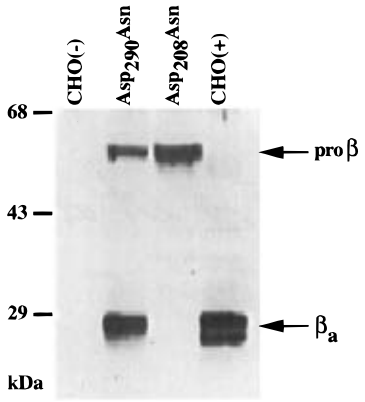

FIGURE 5: Western Blot of lysate from normal (CHO (−)) and permanently transfected CHO cells. Cells were transfected with either the wild type βcDNA (CHO (+)) or mutant βcDNA. The mutations encoded either a Asp^{208}Asn or a Asp^{240}Asn substitution. Lysate protein, 5 μg CHO (+) and 150 μg for the mutants, was varied due to the extremely high levels of expression present in the clone containing the wild type cDNA.

B, selected as a positive control, was unusual in its high level of production (data not shown). As well, since significant levels of the β-precursor (proβ) chain were present in the CHO cells expressing the Asp^{290}Asn-Hex B, it was likely that a smaller percentage of the expressed protein was able to properly fold. Thus, activity measurements (made on the immunoprecipitated human protein) for the Asp^{290}Asn-Hex B had to be adjusted for the lower level of mature β-chain present in the CHO cell clone, before comparing them to the positive control. Densitometry scanning indicated that the clone expressing the mutant βcDNA was producing only 5% of the wild type levels of mature β-chain (Figure 5; Table 5, column 4).

Although dimer formation is thought to be required for exiting of the subunits from the ER, it is definitely required for the expression of Hex activity (Proia et al., 1984). Thus, as a further control, each mutation that produced a lower Hex specific activity was assessed for its degree of dimer formation. This was accomplished by the use of a homobifunctional cross-linking reagent, DSP. Transfected cell lysates were treated with DSP and the denatured (but nonreduced) samples analyzed by Western blotting (Figures 6−8). Analysis of the cross-linked mutant proteins revealed an immunoreactive protein band of about ~130 kDa,

Table 5: Hexosaminidase B Specific Activity in COS Cells Transfected with Wild Type and Mutant Hexosaminidase Constructs

construct[a]	Hex activity[b]	Hex protein % wild type[c]	specific activity[d] (% wild type)
COS (−)	(0.072)	undetectable	0
wild type	19	100	19 (100%)
Asp^{196}Asn	0.33	110	0.31 (1.6%)
Asp^{240}Asn	1.7	110	1.5 (7.8%)
Glu^{253}Gln	17.9	110	16 (82.5%)
CHO (−)	(0.39)	undetectable	0
wild type	104	100	104 (100%)
Asp^{208}Asn	0.01	undetectable	0
Asp^{290}Asn	1.7	4.4	39 (37%)

[a] The constructs encoding these changes were expressed in COS or CHO cells as described under Methods and Materials. [b] Hex activity after immunoprecitation, measured at 1.6 mM MUG, expressed as nmol of MU/(h·μg of lysate protein) with the background, COS (−) or CHO (−) in brackets, subtracted. [c] The relative amounts, as compared to a wild type control, of mature β-protein detected on Western blotting and quantitated by densitometry. This value is only significantly different for permanently transfected CHO cells. [d] Relative specific activity, nmol of MU released/(h·Hex-specific protein in each μg of transfected cell lysate) (see footnote c above).

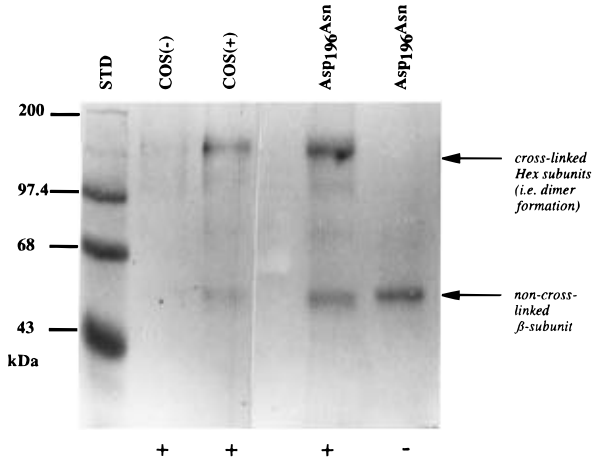

FIGURE 6: Western blots of DSP-treated (cross-linked) extracts from normal COS cells (COS (−)) and those transfected with a wild type (COS (+)) or a mutant βcDNA construct encoding a Asp^{196}Asn substitution (indicated by "+" at the bottom of each lane). Samples of each COS lysate contained equal amounts of nonreduced protein (50 μg). The lysate from cells transfected with these constructs but, treated only with DMSO (indicated by "−" at the bottom of each lane) were used as the negative controls (non-cross-linked, nonreduced β-subunits). Prestained protein M_r standards are shown with their associated M_r (as kDa).

corresponding to the dimerized β-subunits, in all but one case. In the case of the cross-linked β chains carrying the Asp^{208}Asn substitution (Figure 8), which produced only proβ chains (Figure 5), as well as the DMSO-treated, non-cross-linked, negative controls (Figures 6 and 7), only a 65−55 kDa band was evident. This molecular mass corresponds to the monomeric form of the β-subunit (Figures 6−8). We have previously shown that monomeric forms of the wild type enzyme in transfected cells are undetectable by molecular sieve chromatography (Brown et al., 1989). Consequently, the ratio between the bands corresponding to monomers and dimers in the wild type samples reflects the efficiency of the cross-linking reaction. This ratio was similar in all the Hex B mutants except βAsp^{208}Asn and βAsp^{290}Asn, where increased levels of mutant proβ chain were also detected (Figures 5 and 8). Thus, the proportion of mature β subunits, as determined by densitometry, appears

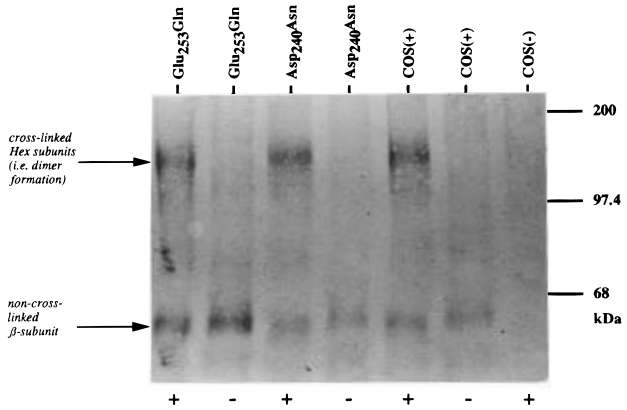

FIGURE 7: Western blots of DSP-treated (cross-linked) extracts from normal COS cells (COS (−)) and those transfected with a wild type (COS (+)) or mutant βcDNA constructs (indicated by "+" at the bottom of each lane). The mutant constructs encoded either a Glu^{253}Gln or a Asp^{240}Asn substitution. Samples of each COS lysate contained equal amounts of nonreduced protein (50 μg). The lysates from cells transfected with these constructs but, treated only with DMSO (indicated by "−" at the bottom of each lane) were used as the negative controls (non-cross-linked, nonreduced β-subunits). The positions of M_r markers are shown on the right.

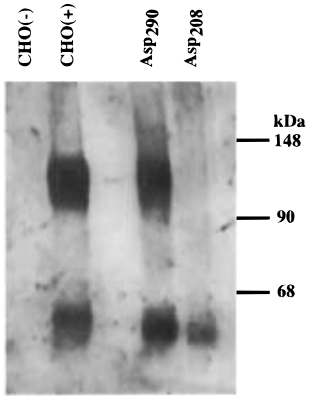

FIGURE 8: Western Blot of DSP-treated (cross-linked), nonreduced extracts from normal (CHO (−)) and permanently transfected CHO cells. Cells were transfected with either the wild type βcDNA (CHO (+)) or mutant βcDNAs. The mutations encoded either a Asp^{208}Asn (Asp^{208}) or a Asp^{240}Asn (Asp^{240}) substitution. Lysate protein was varied, 5 μg CHO (+) and 150 μg for the mutants, due to the extremely high levels of expression present in the clone containing the wild type cDNA. The positions of M_r markers are shown on the right.

to reflect the level of native, dimeric Hex B in transfected cells.

The levels of mature β-chain in each cell lysate was used to calculate the specific activity of each mutant enzyme. As expected, no human Hex activity was detected in CHO cells expressing the Asn^{208}Asn monomeric proβ chain (Figures 5 and 8; Table 5, column 2). The substitution that made the largest change in the specific activity of a mutant Hex B was the Asp^{196}Asn substitution, lowering it to 2% of the wild type value (Table 5, column 4). The Asp^{240}Asn substitution produced Hex B with the second lowest specific activity, 8% of wild type. The Glu^{253}Gln substitution did not significantly affect the specific activity of Hex B, whereas the Asp^{290}Asn mutation lowered the specific activity to 40% (Table 5).

In order to determine if Asp^{196}, Asp^{240}, and/or Asp^{290} are involved in substrate binding or catalysis, the apparent K_m, V_{max}, and k_{cat} values were determined for normal and mutant Hex B using MUG substrate and the solid-state immunoprecipitation assay system (Brown & Mahuran, 1991). Lysates from COS or CHO cells transfected with the wild type cDNA produced an enzyme with an apparent K_m identical to that determined for purified placental Hex B (Table 6). Analysis of the residual activity produced by the Hex B with the Asp^{196}Asn mutation also produced a normal K_m value; however, the k_{cat} value was decreased by 350−910-fold. Thus, the βAsp^{196} residue appears to be involved in catalysis, not in substrate binding. On the other hand, analyses of the kinetic parameters of the Asp^{240}Asn and the Asp^{290}Asn Hex B mutants revealed significant increases in K_m (11- and 3- fold, respectively). The k_{cat} value obtained for the Asp^{240}Asn mutant was also decreased by ∼10-fold, whereas it was near normal for the Asp^{290}Asn Hex B (Table 6). Thus assuming that K_m is essentially equal to K_s for the wild type and mutant enzymes, these residues, particularly Asp^{240}, are likely involved in substrate binding.

Table 6: Kinetic Parameters of Mutant Hex B Proteins with Reduced Specific Activities

construct[a]	K_m[b]	V_{max} [μg of lysate protein][c]	$k_{cat} \times 10^{-6}$ [d] (% of wild type)
placental Hex B	0.71 ± 0.05		3.5
wild type (COS)	0.7 ± 0.1	22 ± 1	3.5[e] (100%)
Asp^{196}Asn	0.7 ±. 0.2	0.05 ± 0.02	0.008 ± 0.003[f] (0.2 ± 0.09%)
Asp^{240}Asn	8 ± 2	3 ± 1	0.4 ± 0.2[f] (11 ± 5%)
wild type (CHO)	0.63 ± 0.09	280 ± 10	3.5[e] (100%)
Asp^{290}Asn	1.9 ± 0.2	8.8 ± 0.4	2.5 ± 0.1[f] (71 ± 3%)

[a] The substitution mutation encoded by the transfected cDNA. [b] mM MUG, kinetic parameters were determined using the immunoprecipitation assay with appropriate negative control values subtracted (for background levels, see Table 5). [c] Maximum nmol of MU released/(h·μg of cell lysate protein). [d] Maximum mol of MU released/(h·mol of Hex B protein) (M_r 130 000). [e] The k_{cat} of the wild type Hex B expressed in the two cell types is assumed to be the same as the purified placental enzyme (Tommasini et al., 1985b). [f] The value was calculated from the predicted k_{cat} of the corresponding wild type positive control and the ratio of the relative abundance of its mature human β protein (obtained from densitometry scanning of Western Blots) as compared to that found in cells expressing the mutant β protein (see Table 5). This calculation is necessary particularly in the case of the permanently transfected CHO cells where there are large variations in transcriptional rates between individual clones.

DISCUSSION

In this report we initially demonstrated, through the chemical modification of Hex with EDC before and after treatment with a strong inhibitor, the presence of at least one protonated, active acidic group in human Hex A and B (Figure 1 and Table 3). Three lines of evidence indicate that such residues should be encoded within exons 5−6 of the *HEX* genes (human β-residues 188−257). First, catalytic residues should be invariant in the aligned sequences from glycosyl hydrolases of the same family as Hex B (family 20) (Henrissat, 1991). This area contains 11 residues that are invariant in the 15 aligned family 20-related sequences (Table 1) as well as 23 sites with conserved substitutions (Figure 2). Outside of this area there are no other aligned invariant residues (Table 4). Part of this region was previously identified by comparing the sequence of human Hex, α-subunit, with that of *Vibrio vulnificus* and *Vibrio harveyi*, corresponding to β-residues 223−254 (Somerville & Colwell, 1993). When compared with the 12 other related sequences (Table 1), there are 4 invariant and 14 conserved residues within this region. However, as shown in the

alignment of all 15 sequences (Figure 2), the area of homology clearly extends back to residue βIle^{189} (seven additional invariant and nine conserved residues). Secondly, one of the two residues associated with the B1 variant form of Tay-Sachs is located in this region. This is the previous discussed (see: the introduction) αArg^{178} (βArg^{211}, exon 5) which is also invariant in the 15 sequences (Figure 2). The other B1 residue, αAsp^{258} (βAsp^{290}), is in exon 7 and is identical in only 10 out of 15 sequences (data not shown). Thirdly, we have prepared α/β fusion cDNAs that exchanged exons 1−4 or 1−7 from α with the aligned areas in β. The $(\alpha^{\text{exons } 1-4}$-$\beta^{\text{exons } 5-14})_2$ enzyme had the substrate specificity and kinetic parameters of Hex B. However, the $(\alpha^{\text{exons } 1-7}$-$\beta^{\text{exons } 8-14})_2$ enzyme was found to contain the α-active site by virtue of its ability to hydrolyze MUGS and G_{M2} ganglioside in the presence of human activator protein (unpublished data).

We examined the effects of a neutral substitution at the two invariant acidic residues in the 15 aligned sequences, βAsp^{196} and βAsp^{208} (Figure 2). We found that βAsp^{196} fit all the requirements for the catalytic protonated acidic residue. Normal levels of mature (lysosomal) β-chain protein were produced from COS cells transfected with a cDNA encoding this substitution (Figure 3), and these subunits were formed into potentially active dimers (Figure 6). However, the residual human Hex B specific activity was reduced to ~2% of normal (Table 5). Kinetic studies of the mutant Hex B showed it to have a normal K_m for MUG with a k_{cat} value only 0.1−0.3% of normal (Table 6). The other invariant residue, Asp^{208}, was shown to be very important in the initial folding and/or dimer formation of the proβ subunit. These events are prerequisite for exit from the ER (Proia et al., 1984). Western blots detected only the proβ form of the subunit (Figure 5), and cross-linking studies detected only proβ monomers (Figure 8).

We also examined less well conserved residues. Asp^{240} is identical in 14 sequences and substituted with Glu in one. We show that this residue is important in the binding of the MUG substrate. The K_m of the mutant Hex B was increased by 11-fold, whereas the k_{cat} was reduced by a factor of 10 (Table 6). Asp^{290} is less well conserved being identical in 10/15 sequences but being substituted in the remaining 5 by either Arg, Val, Ser (X2), or Thr (data not shown), but its α homologue has been associated with the B1 variant of Tay-Sachs disease (Fernandes et al., 1992). Substitution of this residue with a conservative Asn indicates that it is likely involved both in substrate binding and protein folding. Significant levels of proβ chain were found in CHO cell lysates (Figure 5), and there appeared to be a decrease in the ratio between dimers and monomers in our cross-linking study (Figure 8). The K_m for MUG was increased 3-fold, whereas the k_{cat} (based on the level of mature β-chain as determined by densitometry) was only slightly affected (Table 6). The fact that in the patient with the B1 variant form of Tay-Sachs disease this residue is substituted with a much less conservative His residue may have added to the severity of the mutation's effect on both of these properties. We investigated one other residue, βGlu^{253}, which was the least conserved of the group, identical in 8/15 (Figure 2). Substitution of this residue with Gln produced a Hex B with a normal specific activity (Table 5).

One other acidic residue has recently been linked to the active site of Hex B. This residue, βGlu^{355}, was identified through its ability to specifically bind a photoaffinity label. Unfortunately, no mutational analysis was done to confirm the role of this residue in the active site of Hex B, and the label was placed at the aglyco region of the inhibitor, an area where Hex shows little or no specificity (Liessem et al., 1995). Because this residue is not invariant, it fails to fit our criteria as a candidate catalytic site residue, although it still could be involved in substrate binding. It is identical in 10/15 of the aligned sequences and substituted with either Val (X2), Asn (X2), or Glu in the others (data not shown). It also does not reside in the area of greatest homology within the primary structures of other family 20 enzymes (Table 4 and Figure 2).

It is thought that all glycosyl hydrolases act by a general acid catalysis mechanism in which two acidic amino acids, one protonated the other one not, participate in a single- or double-displacement reaction. Furthermore, when hydrolases are grouped into evolutionarily related families, the active acidic residues are invariant within the aligned sequences of the family members; Hex has been placed in family 20 (Henrissat, 1991). Alignments of 15 members of family 20 revealed only two invariant acidic residues, βAsp^{196} and βAsp^{208} (Figure 2).

Two recent articles have challenged the need of the nucleophilic (unprotonated) acid residue for glycosidases whose substrates are restricted to those containing an acetamido group. First, it was found that the compound (±)-6-acetamido-1,2-anhydro-6-deoxymyoinositol (*N*-acetylconduramine B *trans*-epoxide) is a tight-binding inhibitor and pseudosubstrate for *N*-acetyl-β-glucosaminidases, i.e., Hex, from both bovine kidney and jack bean. With other glycosidases their unprotonated active acidic residue forms an ester bond with the activated (by the proton transferred from the protonated acidic residue) oxirane intermediate of this type of inhibitor. The unprotonated acid residue normally stabilizes the substrate's transient carbonium ion intermediate for the hydrolysis of the β-1,4-glycosidic bonds. Since this inhibitor did not form a covalent bond with Hex, the authors conclude that a carbonium ion intermediate (at C-1) was not part of its catalytic mechanism. They proposed that the unprotonated acid group is replaced in Hex by an unidentified active basic group (kept unprotonated by the microenvironment of the active site) which removes a proton from the acetamido group of the bound substrate (or their inhibitor) producing an internal oxazoline (C-1 joined to the former acetamido oxygen) structure as a transition state or reactive intermediate (Legler & Bollhagen, 1992). In the second report the same type of internal oxazolinium intermediate was demonstrated for the family 18 plant chitinase/lysozyme, hevamine, by determining its X-ray structure with a bound inhibitor, allosamidin (van Scheltinga et al., 1995). These authors also point out that such a stabilization mechanism, called anchimeric assistance or neighboring group participation, is well known in organic chemistry and explains why the spontaneous hydrolysis of methyl-2-acetamido-2-deoxy-β-D-glucopyranoside is 1000-fold enhanced over that of methyl β-D-glucopyranoside. The data presented in this report add further support to this mechanism of "substrate-assisted catalysis" in Hex. We have demonstrated that of the two invariant acidic residues, βAsp^{196} and βAsp^{208} (Figure 2), only one, βAsp^{196}, is involved in substrate catalysis (Tables 5 and 6). Thus, βAsp^{196} is likely the active protonated acid group in Hex.

Site-directed mutagenesis studies of two other glycosyl hydrolases indicate that activity is lowered to near the level

of detectablity if the protonated acid group is replaced by a neutral residue. In the case of chicken egg white lysozyme (family 22) a $Glu^{35}Gln$ substitution reduced specific activity to 0.1% ± 0.1% of the wild type level (Malcolm et al., 1989), and in an exoglucanase/xylanase CenA from *Cellulomonas fimi* (family 10) the k_{cat}/K_m of an $Glu^{127}Ala$ substituted enzyme was reduced over 6000-fold as compared to the wild type enzyme (MacLeod et al., 1994). The specific activity and k_{cat} data we present here for the $\beta Asp^{196}Asn$ substituted Hex B appear to be higher than might be expected based on these examples. However, the pK_a of the substrate's aglycon affects the degree to which k_{cat} is lowered when the acid catalyst is neutralized, i.e., the higher the pK_a the poorer the leaving group and the larger the fold decrease in activity (MacLeod et al., 1994; Malcolm et al., 1989). MU is a relatively good leaving group as compared to the β-linked sugar moieties present on natural substrates, and the acidic pH at which Hex hydrolyzes MUG should also decrease the role of the protonated acid group.

Our previous data identifying βArg^{211} as an active residue in Hex B (see the introduction) (Brown & Mahuran, 1991) have also been strengthened by our present observation that it is an invariant residue in the 15 Hex-related sequences identified (Figure 2). Given its high pK_a, it is unlikely to be the catalytic basic residue discussed above. Its most likely role would be in controlling the pK_a of an active acidic residue such as βAsp^{196}.

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BI960246+