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Identification of Candidate Active Site Residues in Lysosomal β-Hexosaminidase A*

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The β-hexosaminidases (Hex) catalyze the cleavage of terminal amino sugars on a broad spectrum of glycoconjugates. The major Hex isozymes in humans, Hex A, a heterodimer of α and β subunits (αβ), and Hex B, a homodimer of β subunits (ββ), have different substrate specificities. The β subunit (*HEXB* gene product), hydrolyzes neutral substrates. The α subunit (*HEXA* gene product), hydrolyzes both neutral and charged substrates. Only Hex A is able to hydrolyze the most important natural substrate, the acidic glycolipid GM_2 ganglioside. Mutations in the *HEXA* gene cause Tay-Sachs disease (TSD), a GM_2 ganglioside storage disorder. We investigated the role of putative active site residues Asp-α258, Glu-α307, Glu-α323, and Glu-α462 in the α subunit of Hex A. A mutation at codon 258 which we described was associated with the TSD B1 phenotype, characterized by the presence of normal amounts of mature but catalytically inactive enzyme. TSD-B1 mutations are believed to involve substitutions of residues at the enzyme active site. Glu-α307, Glu-α323, and Glu-α462 were predicted to be active site residues by homology studies and hydrophobic cluster analysis. We used site-directed mutagenesis and expression in a novel transformed human fetal TSD neuroglial (TSD-NG) cell line (with very low levels of endogenous Hex A activity), to study the effects of mutation at candidate active site residues. Mutant *HEXA* cDNAs carrying conservative or isofunctional substitutions at these positions were expressed in TSD-NG cells. αE323D, αE462D, and αD258N cDNAs produced normally processed peptide chains with drastically reduced activity toward the α subunit-specific substrate 4MUGS. The αE307D cDNA produced a precursor peptide with significant catalytic activity. Kinetic analysis of enzymes carrying mutations at Glu-α323 and Asp-α258 (reported earlier by Bayleran, J., Hechtman, P., Kolodny, E., and Kaback, M. (1987) *Am. J. Hum. Genet.* 41, 532–548) indicated no significant change in substrate binding properties. Our data, viewed in the context of homology studies and modeling, and studies with suicide substrates, suggest that Glu-α323 and Asp-α258 are active site residues and that Glu-α323 is involved in catalysis.

The β-hexosaminidases (Hex[1], EC 3.2.1.52) are lysosomal hydrolases that catalyze the cleavage of terminal β-*N*-acetylglucosamine or β-*N*-acetylgalactosamine residues on a broad spectrum of glycoconjugates. The major Hex isozymes in humans are: Hex A, a heterodimer composed of one α and one β subunit and Hex B, a homodimer of two β subunits. A third isozyme, Hex S, is composed of two α subunits, which are unstable and not normally found in most tissues. The α and β subunits are structurally related, sharing 60% amino acid identity in the mature form (1, 2). Both subunits are catalytically active with different but overlapping substrate specificities (3). The β subunit, in Hex A and Hex B, hydrolyzes neutral substrates, whereas the α subunit, in Hex A and Hex S, hydrolyzes neutral substrates as well as substrates bearing a negative charge either on the terminal sugar (*e.g.* GlcNac-SO_4) or on a distinct residue (*e.g.* sialic acid) (4). The latter includes the most important natural substrate, the sialic-acid containing glycosphingiolipid, GM_2 ganglioside, found mainly in neuronal tissue. Only Hex A catalyzes cleavage of the terminal β-*N*-acetylgalactosamine on GM_2 ganglioside in the presence of the substrate-specific protein cofactor, the GM_2-activator protein (5).

Mutations in the *HEXA*, *HEXB*, and *GM2A* genes, encoding the α and β subunits of Hex A and the GM_2 activator, respectively, lead to a group of inherited neurodegenerative diseases, collectively known as the GM_2 gangliosidoses, that are characterized by lysosomal accumulation of GM_2 ganglioside mainly in neuronal tissue. These disorders range in severity from Tay-Sachs disease (TSD), a progressive and fatal neurodegenerative disorder of infancy, to clinically milder or later onset forms of GM_2 gangliosidosis occurring in patients with some residual enzyme activity (reviewed in Gravel *et al.* (6)).

Although more than 70 mutations at the human *HEXA* locus, and 14 at the *HEXB* locus have been described (6), few have revealed information about the location or properties of the α and β subunit active sites. A subset of *HEXA* mutations, known as the B1 mutations, lead to production of normal amounts of mature Hex A, which is deficient in α subunit catalytic activity without affecting β subunit activity (7). The B1 mutations are thus compatible with normal maturation of Hex subunits and delivery of structurally intact Hex A to the lysosome. The presence of a mature Hex A, unable to hydrolyze charged substrates, suggested that the B1 biochemical pheno-

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[1] The abbreviations used are: Hex, hexosaminidase; TSD, Tay-Sachs disease; *HEXA*, *HEXB*, and *GM2A*, the genes for the α and β subunits of Hex A and the GM_2 ganglioside activator protein, respectively; 4MU, 4-methylumbelliferone; 4MUG, 4-methylumbelliferone β-*N*-acetylglucosamine; 4MUGS, 4-methylumbelliferone *N*-acetylglucosamine-6-sulfate; NG, neuroglial; PCR, polymerase chain reaction.

type might be associated with mutations at or near the α subunit active site (4, 7, 8). The first B1 mutations described were αR178H and αR178C (7–9). *In vitro* generated mutation at the homologous site to Arg-α178 in the β subunit, Arg-β211, resulted in production of mature Hex B devoid of catalytic activity (10). More recently, we identified a third B1 mutation, αD258H (11). Both Arg-α178 and Asp-α258 have been proposed as candidates for participation at or near the active site of the α subunit.

The mechanism of cleavage of glycosidic bonds by Hex remains unclear. Sinnott (12) has proposed that glycosyl hydrolases employ an acid-catalysis mechanism involving the participation of two acidic residues (a proton donor and a nucleophile) at the active site, as identified in glycoside cleavage by β-glucosidases (13–16), α-glucosidases (17), β-glucanases (18–20), β-galactosidases (21–23), and chitinases (24, 25) and the β-*N*-acetylglucosaminidase (chitobiase) (26). In contrast, based on inhibition studies using nitrogen-containing substrate inhibitors, Legler and Boothagen (27) and Legler *et al.* (28) proposed that the mechanism of Hex catalysis involves both an acidic and a basic residue and depends on a transition state in which the glycosidic O of the substrate is joined with the acetyl group of the pyranose ring, a mechanism fundamentally different from that of other glycosidases.

In this study, we investigated candidate active site residues in the α subunit of Hex A through analysis of mutations that affect catalytic activity without disrupting maturation of the enzyme. We introduced conservative mutations at residue Asp-α258, as well as at three glutamic acid residues (Glu-α307, Glu-α323, and Glu-α462) which are evolutionarily invariant in family 20 of glycosyl hydrolases using the classification system of Henrissat (29). We then studied the impact of these substitutions on enzyme activity and maturation after transfection of mutant cDNAs using an SV40-transformed neuroglial (NG) cell line established from a fetus with Tay-Sachs disease which is devoid of endogenously expressed α subunits but produces functional β subunits.

EXPERIMENTAL PROCEDURES

Cell Culture—NG141 (NG) and NG125 (TSD-NG), SV40-transformed fetal TSD and normal neuroglial cell lines, respectively, were provided by L. Hoffman and S. Brooks (Kingsbrook Jewish Medical Center, Brooklyn, NY). Cells were cultured in α-minimum essential medium with 15% fetal calf serum and antibiotics.

Recombinant Plasmids—The *Escherichia coli* β-galactosidase gene, in pSVLβgal (Clontech), was used as a reporter gene to control for transfection efficiency. The cloned human *HEXA* cDNA (1) was inserted into pRCCMV and pREP4 (Invitrogen) to produce pRCCMVα and pREP4α. Preparation of PRCCMVα involved: 1) subcloning the *HEXA* cDNA from PSVLα into pCRII (Invitrogen) as an *Xho*I/*Bam*HI fragment, 2) subcloning of an *Nsi*I *HEXA* cDNA-containing fragment PCRII subclone into the *Pst*I site of pBluescript (Stratagene), 3) subcloning an *Xho*I/*Xba*I fragment from pBluescript into a PCRII intermediate, and 4) cloning a *Not*I/*Xba*I *HEXA* cDNA-containing fragment from the PCRII subclone into PRCCMV. Preparation of pREP4α involved cloning the *Xho*I/*Bam*HI *HEXA* cDNA-containing fragment from PSVLα into pREP4. Construction of pCMVα required creating a *HEXA* cDNA flanked by *Not*I sites. The *HEXA* cDNA insert in pRCCMVα has a *Not*I site at the 5′ end of the gene. This insert was subcloned into the *Bam*HI site of pBluescript (KS−, Stratagene) introducing a second *Not*I site downstream from the 3′ end of the gene. This step permitted the cloning of the insert into the unique *Not*I site of pCMVα. All plasmids were purified on Qiagen columns prior to transfection.

Site-directed Mutagenesis—Mutations were introduced into the *HEXA* cDNA using a modified protocol of the Clontech Transformer Mutagenesis Kit. The second screening step for mutant clones was omitted. Mutant clones were identified after the first screen by PCR amplification of samples of isolated disrupted bacterial colonies followed by restriction enzyme digestion or allele-specific hybridization of amplified product to identify mutant genotypes. Mutations were introduced into pSVLα, a cassette containing the altered sequence was subcloned into pBS(KS)*HEXA*, and the full-length mutant cDNA was subsequently subcloned into pCMV. Mutant pCMVα inserts were sequenced (Pharmacia T7 sequencing kit) and the plasmids purified on Qiagen columns prior to transfection.

Transfection—For transient expression in cell line NG 125, subconfluent T175s (175 cm^2, Sarstedt) were harvested by trypsinization and washed twice with 1 × phosphate-buffered saline. The cell pellet was resuspended in Optimem medium (Life Technologies, Inc., containing 5% fetal bovine serum) to obtain a final concentration of 6×10^6 cells/ml. Cell suspension (800 μl), 20 μg of pCMVα, and 2 μg of pSVLβgal were placed in a 0.4-cm cuvette, mixed, placed on ice for 5 min, and pulsed (500 microfarads, 400 V) using a Bio-Rad electroporation apparatus. The time constant was between 16 and 18 ms. After 15 min on ice, 800 μl of α-minimum essential medium (without antibiotics) was added to the suspension. For the β-galactosidase qualitative assay, 250 μl of transfected cell suspension were plated on 12 multiwell plates. The remainder of the transfected cell suspension was grown for 48 h in a T75 (75 cm^2).

Enzyme and Protein Assays—A qualitative β-galactosidase assay was performed to determine the percentage of surviving cells which expressed bacterial enzyme. Multiwell-plated cells were incubated as described by Lake (30). After 24-h incubations the number of blue cells was estimated microscopically. Harvested cells were lysed by freeze-thawing in 0.25 M Tris-HCl (pH 7.4) and protein determined by the Bradford method (Bio-Rad). A fluorescent assay (31) was adapted for quantitation of β-galactosidase activity in transfected cell lysates. The reaction mixture contained 3 μl of 100 × magnesium solution (4.5 M 2-mercaptoethanol, 0.1 M $MgCl_2$), 100 μl of 0.5 mM 4 methyl-umbelliferyl β-D-galactoside, and approximately 2–5 μg of lysate protein in 0.1 M sodium phosphate buffer (pH 7.5) in a volume of 334 ml. After incubation at 37 °C for 15 min, fluorescence, due to release of 4MU, was determined using a Perkin-Elmer spectrofluorimeter (excitation wavelength, 360 nm; emission, 447 nm). Hexosaminidase activity was also determined fluorometrically using either 4MUGS (α subunit substrate) (32) or the 4MUG (β subunit) substrate.

Western Blot Analysis—The enhanced chemiluminescence (ECL) Western blotting kit from Amersham Corp. was used to detect the presence of the Hex A α subunit with a polyclonal rabbit anti-human Hex A antibody. Both the primary and secondary (rabbit Ig, horseradish peroxidase-labeled antibody) antibodies were used at a 1:5000 dilution.

Chromatofocusing of Transfected Cell Extracts—The hexosaminidase isoenzyme profile in cell lysates was determined by chromatofocusing using the Pharmacia Polybuffer Exchanger (PEB) system according to a modified protocol of O'Dowd *et al.* (33). Transfected cell extracts were freeze-thawed (three times) in 0.025 M imidazole buffer (pH 7.4), and the protein concentration was determined by the Bradford method. All steps were carried out at 7 °C. Approximately 1 ml of PEB74 slurry was used to prepare a column in a 1-ml syringe. The column was washed with the equivalent of 1.5–2 × bed volume with 0.025 M imidazole. The protein extract was added after passing 2 × 100 μl of Polybuffer (pH 4.0) through the column. Four hundred-microliter fractions were collected. Sodium citrate buffer (0.13 M, pH 3.46) was used to elute Hex S after a pH of 4.0 was reached using a pH gradient.

Kinetic Analysis—The K_m and K_i for wild type and mutant Hex A proteins were determined using 4MUGS substrate at concentrations from 1.0 to 7.5 mM. For inhibition studies, the competitive inhibitor *N*-aetylglucosamine-6-phosphate (Sigma) was added to a final concentration of 10 mM.

RESULTS

Expression of the HEXA Gene in TSD-NG Cells—The endogenous activity of NG cells toward the α-specific substrate 4MUGS was 715 ± 13 nmol/mg/h. In untransfected TSD-NG cells, the rate of 4MUGS hydrolysis is <1% of that in NG cells. This trace activity is probably due to the residual action of Hex B on 4MUGS (32).

In order to maximize α subunit expression through transfection, several vectors carrying the *HEXA* gene cDNA were assessed for their ability to drive expression of enzymatic activity when transfected into TSD-NG cells. Transfection efficiency (percent surviving cells catalyzing 5-bromo-4-chloro-3-indoyl β-D-galactoside hydrolysis) was determined to be 10–20%. Hexosaminidase activity in cells transfected with 20 μg of plasmids pSVLα, pRCCMVα, pREP4α, or pCMVα was at least 10-fold higher than activity in mock-transfected cells (Table I). The highest level of expression (approximately 1000 × mock-trans-

TABLE I
HEXA gene expression in TSD-NG cells

Vector	Time harvested[a]	Hex activity[b]
	h	*nmol/h/mg*
Untransfected NG cells		715 (±13)
Untransfected TSD-NG cells		3 (±2)
pSVLα[c]	48	43 (±15)
pRCCMVα	48	358 (±52)
pREP4α	48	611 (±12)
pCMVα	24	1825 (±246)
pCMVα	48	3986 (±529)
pCMVα	72	7145 (±365)

[a] Hours post-transfection.
[b] Nanomoles of 4MUGS hydrolyzed/h/mg of protein.
[c] All transfections into TSD-NG cells.

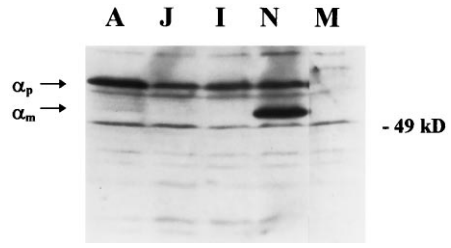

FIG. 1. **Western blot analysis of TSD-NG cells transfected with *A*, pCMVαG269S (*HEXA* mutation associated with adult onset TSD); *J*, pCMVαG250D (*HEXA* mutation associated with juvenile onset TSD); *I*, pCMVαR170W (*HEXA* mutation associated with infantile classical TSD); and *N*, pCMVα (wild type α subunit).** α_p and α_m indicate precursor and mature forms of Hex A α subunit. *Lane M* represents mock-transfected cells.

fected cells) was achieved when cells were transfected with pCMVα. Further analysis of Hex activity in TSD-NG cells at 24, 48, and 72 h post-transfection with pCMVα (Table I) showed that activity continued to increase throughout the 72-h period. The pCMVα plasmid expressed over a 48-h incubation period post-transfection was selected for all subsequent experiments.

Western blot analysis of transfected (pCMVα and pSVLβgal) and mock-transfected (pSVLβgal) cell extracts confirmed that the increase in 4MUGS activity in TSD-NG cells is associated with α subunit expression (Fig. 1, *lane N*). Both precursor and mature α subunit are absent in mock-transfected TSD-NG cell extracts (Fig. 1, *lane M*) and present in cells transfected with pCMVα.

In order to determine whether the α subunits encoded by the *HEXA* cDNA were expressed as the heterodimeric enzyme Hex A (αβ) or the homodimeric species Hex S (αα), the Hex isozyme forms in transfected TSD-NG cell lysates were resolved by chromatofocusing. Fig. 2*a* illustrates the chromatofocusing profile of TSD-NG cells transfected with pCMVα and assayed with 4MUG (all Hex) and 4MUGS (Hex A and Hex S) to detect all Hex isozyme species. All three hexosaminidase isoenzymes were present, and eluted at their expected pIs. Hex S was the isoenzyme present in greatest abundance followed by Hex A and Hex B respectively. This high expression of the nonphysiological Hex S in TSD-NG cells is the most likely due to the massive overexpression of α subunits in the face of limiting, endogenously produced β subunits. Total Hex activity in pCMVα transfected TSD-NG cells (harvested at 48 h post-transfection) is 5–6-fold greater than endogenous Hex activity in untransfected normal NG cells (Table I). Given an efficiency of transfection of 10–20%, the transfected cells express up to 60-fold greater Hex activity than normal untransfected NG cells. Despite overwhelming α subunit synthesis, a significant proportion (22%) of the transfected gene product is expressed as the Hex A heterodimer as shown by the chromatofocusing profile (Fig. 2).

Expression of HEXA Mutations—In order to evaluate the expression of mutant α subunits in TSD-NG cells, we initially examined mutations known to cause the infantile acute (αR170W) (11) subacute (αG250D) (35) and chronic (αG269S) (37) forms of GM_2 gangliosidosis. The mutant cDNAs were expressed in TSD-NG cells (Table II) and cell lysates assayed for Hex A specific activity using 4MUGS. Hex A activity in lysates of αG269S- and αG250D-transfected TSD-NG cells was <4% and 1.5%, respectively, of the activity measured after transfection with wild type pCMVα. These Hex A activities were 9- and 4-fold greater than mock-transfected activity measured with the 4MUGS substrate. In contrast, expression of αR170W exhibited no significant 4MUGS activity above that of mock transfected cells. Western blot analysis showed that the α subunit synthesized by all three mutant cDNAs appeared in the precursor form (Fig. 1, *lanes A*, *J*, and *I*). Only the αG269S mutation appeared to be associated with expression of a small amount of mature α subunit (Fig. 1, *lane A*). These results are compatible with previous findings on αG269S (34) and αG250D (35) in COS cell expression studies and demonstrate complete inactivation of Hex A by the αR170W mutation consistent with the infantile TSD phenotype. In the latter case, the R170W substitution is associated with expression of an α subunit precursor (Fig. 1, *lane I*) but not with its maturation and targeting to the lysosome.

Analysis of Putative Active Site Residues—We next examined the expression in TSD-NG cells of mutations at amino acid residues which are candidates for participation in the Hex A α subunit catalytic site. To assess the role of mutation at residue Asp-α258 (αD258H), first described in association with the B1 biochemical phenotype of TSD, *HEXA* cDNA constructs carrying the conservative or isosteric substitutions αD258H, αD258E, or αD258N were expressed in TSD-NG cells and Hex A activity measured in transfected cell lysates. All three mutations resulted in negligible or trace amounts of Hex A activity, with αD258N exhibiting 2-fold background and the others the same activity as measured in mock-transfected cells (Table III, Experiment 1). Western blot analysis of transfected cell extracts (Fig. 3), revealed that maturation of pro-α chains carrying the αD258H mutation was reduced. In contrast, expression αD258N and αD258E mutant cDNAs resulted in production of mature α subunit. Chromatofocusing of αD258N cell extracts revealed that this mutation significantly reduced 4MUGS hydrolysis by Hex A and Hex S (data not shown). The 4MUG hydrolysis of Hex A was reduced to a lesser extent. This result was expected since 4MUG hydrolysis is catalyzed by both the α and β subunits of Hex A.

We then studied the effects of mutation at conserved residues Glu-α307, Glu-α323, and Glu-α462, predicted as candidate active site residues by homology studies and hydrophobic cluster analysis of family 20 glycosyl hydrolases. Fig. 4, *a* and *b*, illustrate multiple alignment of the two regions of family 20 enzymes which produce significant alignment. *Asterisks* indicate the three evolutionarily invariant glutamate residues identified as candidate active site residues using the classification system of Henrissat. Other invariant amino acids are also indicated.

HEXA cDNA constructs carrying the conservative substitutions αE307D, αE323D, or αE462D were expressed in TSD-NG cells and Hex activity measured in transfected cell lysates. All three substitutions had dramatic effects on catalytic activity (Table III, Experiment 2). αE323D did not exhibit any 4MUGS activity above background whereas αE462D and αE307D exhibited 14- and 60-fold 4MUGS activity above the background activity obtained in mock transfected cells respectively. West-

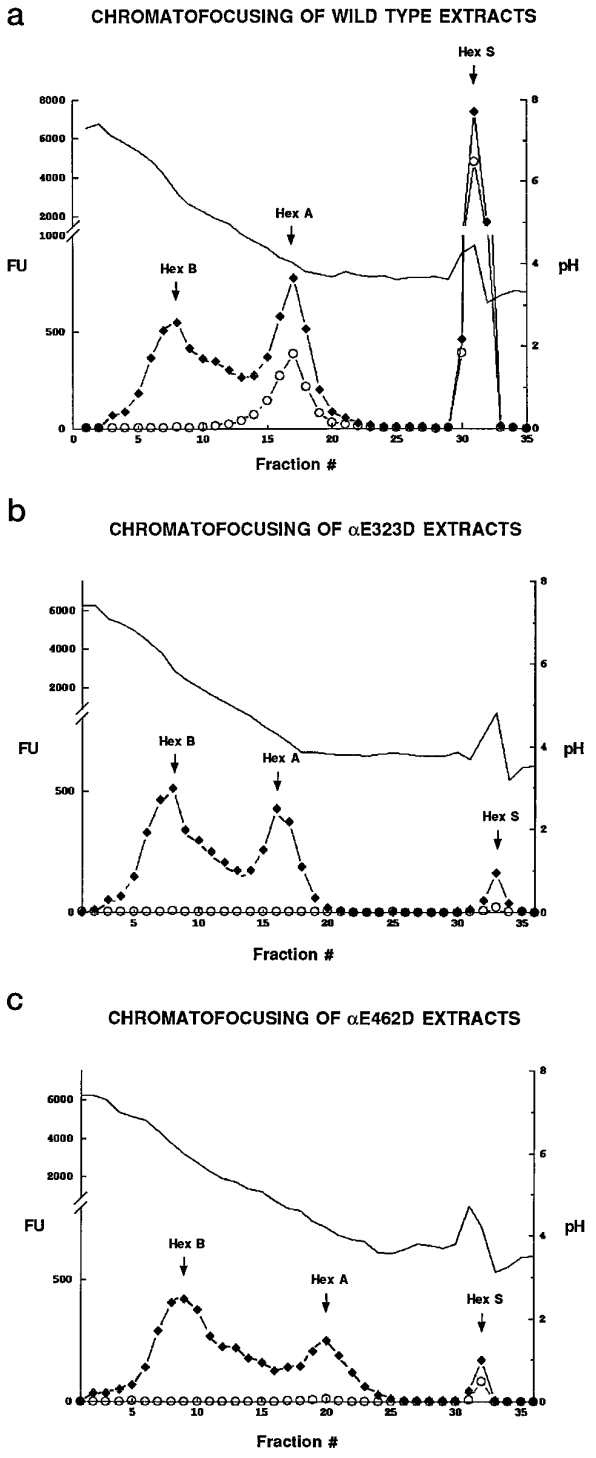

FIG. 2. **Chromatofocusing of NG cell extracts transfected with pCMV*α*, pCMV*α*E323D, and pCMV*α*E462D and assayed with the synthetic substrates 4MUG (■[sbond—■) and 4MUGS** (○——○). Activity peaks for Hex B, Hex A, and Hex S are indicated. (——) pH gradient.

ern blot analysis of transfected cell extracts showed that only the αE323D and αE462D mutations were compatible with synthesis of significant amounts of precursor and mature α subunit (Fig. 5). Chromatofocusing of αE323D and αE462D cell extracts confirmed that the Hex A isoenzyme is processed normally but has a catalytically defective α subunit that is unable to hydrolyze 4MUG or 4MUGS (Fig. 2, *b* and *c*). The αE307D mutation, however, dramatically reduces α subunit maturation.

TABLE II
Expression of GM2 gangliosidosis mutations

Vector	Age of onset of TSD[a]	Hex/β-galactosidase activity[b]
pCMVα	Normal	49.0 (±15)
pCMVαG269S	Adult	1.5 (±0.8)
pCMVαG250D	Juvenile	0.67 (±0.3)
pCMVαR170W	Infantile	0.20 (±0.08)
Mock		0.16 (±0.02)

[a] Age of onset of TSD in patients carrying indicated mutations.
[b] The numbers express a ratio. Hex and β-galactosidase activity were expressed as nanomoles/h/mg of protein.

TABLE III
Effect of substitutions at putative active site residues on Hex A activity

Genotype	Hex/β-galactosidase ratio
Experiment 1	
Wild type	88.0 (±32)
αD258H	0.15 (±0.04)
αD258E	0.15 (±0.05)
αD258N	0.37 (±0.06)
Mock	0.13 (±0.07)
Experiment 2	
Wild type	36 (±14)
αE307D	3.6 (±0.5)
αE323D	0.2 (±0.09)
αE462D	0.85 (±0.13)
Mock	0.06

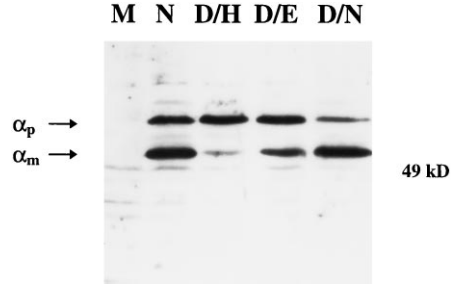

FIG. 3. **Western blot analysis of TSD-NG cells transfected with pCMVαD258H (*D/H*), pCMVαD258E (*D/E*), pCMVαD258N (*D/N*,) and pCMVα (*N*).** *Lane M* represents mock-transfected cells.

Kinetic experiments were performed to evaluate the impact of the αE323D mutation on the binding affinity of Hex A for the substrates 4MUGS and for the competitive inhibitor *N*-acetylglucosamine-6-PO_4 (Fig. 6). The Lineweaver-Burke plots for the wild type enzyme are monophasic, representing the contribution of a single active site to the hydrolysis of 4MUGS. In contrast, the kinetics of hydrolysis of this substrate by the Hex A αE323D enzyme are biphasic revealing a significant contribution of a second active site (the β subunit) at high substrate concentrations. When the kinetic parameters of the high affinity site are evaluated the K_m for 4MUGS is 2.5 mM for Hex A αE323D compared to 1.7 mM for the wild type enzyme. Similiarly K_i values for the competitive inhibitor are 15.6 mM for the αE323D-substituted Hex A and 11.0 mM for wild type Hex A. We showed, previously, that the K_m for Hex A in fibroblasts from a patient carrying the D258H allele (and a null allele) was identical to that of wild type enzyme (7).

DISCUSSION

β-Hexosaminidase catalyzes the hydrolysis of its glycoside substrates by a "retaining" mechanism in which the anomeric configuration of the substrate remains unchanged (12, 36). Most retaining glycosidases employ an acid catalysis mechanism in which two amino acids function respectively as proton donor and nucleophile (12). Alternatively, glycosaminidases may employ a second mechanism, termed substrate-assisted catalysis, in which an acidic residue and a basic residue par-

a

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HEXA_HUMAN    WGALRGLETFSQLV-WKSAEGTFFI-----NKTEIEDFPRFPHRGLLLDT    176
HEXA_MOUSE    WGALRGLETFSQLV-WKSAEGTFFI-----NKTKIKDFPRFPHRGVLLDT    176
HEXB_HUMAN    WGALRGLETFSQLV-YQDSYGTFTI-----NESTIIDSPRFSHRGILIDT    209
HEXB_MOUSE    WGALRGLETFSQLV-YQDSFGTFTI-----NESSIADSPRFPHRGILIDT    188
HEXB_FELCA    WGVLRGLETFSQLI-YQDSYGTFTV-----NESDIIDSPRFPHRGILIDT    151
HEXA_DICDI    YGAMRGLETFKQLIVYNELENSYSI-----VCVSISDSPRYPWRGFMVDS    164
HEX1_ENTHI    YGARHAFETLLQLIRISSNKFVISQ-----LPIKISDAPRFKWRGLMVDP    150
HEX1_CANAL    WGALHGLVSLQQLIIHTS-EDKYVV----PSSVTISDFPNFKHRGLMIDS    175
HEXC_BOMMO    FGVRNGLETLSQLIVYDDIRNNLLI----VRDVTIKDRPVYPYRGILLDT    220
CHB_VIBHA     AGAFYAVQSIFGLVDSQNADS--------LPQLSIKDAPRFDYRGVMVDV    341
CHB_SERMA     AGVFYGLQSILSLVPSDGSGK--------IATLDASDAPRFPYRGIFLDV    347
HEX_VIBVU     AGAFYGVQSLAGLVTV-GKDT--------INQVSINDEPRLDYRGMHMDV    324
HEXB_ALTSO    AAAFYALQSLAGLLDINDL-R--------IPMVDIIDTPRYDFRGLHVDV    336
HEXA_PORGI    HGAFYGMQTLLQLLPAEVESSNEVLLPMTVPGVEIKDEPAFGYRGFMLDV    179
                          .                       . .    ..   .

HEXA_HUMAN    SRHYLPLSSILDTLDVMAYNKLNVFHWHLVDDPSFPYESFTFPELMRKGS    226
HEXA_MOUSE    SRHYLPLSSILDTLDVMAYNKFNVFHWHLVDDSSFPYESFTFPELTRKGS    226
HEXB_HUMAN    SRHYLPVKIILKTLDAMAFNKFNVLHWHIVDDQSFPYQSITFPELSNKGS    259
HEXB_MOUSE    SRHLLPVKTIFKTLDAMAFNKFNVLHWHIVDDQSFPYQSTTFPELSNKGS    238
HEXB_FELCA    ARHFLPVKSILKTLDAMAFNKFNVLHWHIVDDQSFPYQSVTFPELSNKGS    201
HEXA_DICDI    ARHYIPKNMILHMIDSLGFSKFNTLHWHMVDAVAFPVESTTYPDLT-KGA    213
HEX1_ENTHI    SRNPLSPLMFKRIIDTLASVKANVLHIHLSDAQTFVFESKKYPLLHQKGM    200
HEX1_CANAL    GRNFLTVDSILEQIDIMALSKMNSLHWHLADSQSWPVALESYPHMIK-DA    224
HEXC_BOMMO    ARNFYSIDSIKRTIDAMAAVKLNTFHWHITDSQSFPLVLQKRPNLSKLGA    270
CHB_VIBHA     ARNFHSKDAILATLDQMAAYKMNKLHLHLTDDEGWRLEIPGLPELTEVGA    391
CHB_SERMA     ARNFHKKDAVLRLLDQMAAYKLNKFHFHLSDDEGWRIEIPGLPELTEVGG    397
HEX_VIBVU     SRNFHSKELVFRFLDQMAAYKMNKFHFHLADDEGWRLEINGLPELTQVGA    374
HEXB_ALTSO    ARNFRSKAFILQTIEQMAAYKLNKLHLHLADDEGWRLAIDGLDELTSVGA    386
HEXA_PORGI    CRHFLSVEDIKKHIDIMAMFKINRFHWHLTEDQAWRIEIKKYPRLTEVGS    229
               ■                  . .  . .

HEXA_HUMAN    Y----------------------NPVTHIYTAQDVKEVIEYARLRGIRVL    254
HEXA_MOUSE    F----------------------NPVTHIYTAQDVKEVIEYARLRGIRVL    254
HEXB_HUMAN    Y----------------------S-LSHVYTPNDVRMVIEYARLRGIRVL    286
HEXB_MOUSE    Y----------------------S-LSHVYTPNDVRMVLEYARLRGIRVI    265
HEXB_FELCA    Y----------------------S-LSHVYTPNDVHTVIEYARLRGIRVI    228
HEXA_DICDI    F----------------------SP-SATFSHDDIQEVVAYAKTYGIRVI    240
HEX1_ENTHI    Y----------------------DE-SFVLTQSFLRELAQYGANRGVIVY    227
HEX1_CANAL    Y----------------------SN-DEVYSKNDLKYIVDYARARGVRVI    251
HEXC_BOMMO    Y----------------------SP-TKVYTKQDIREVVEYGLERGVRVL    297
CHB_VIBHA     NRCFDTQEKSCLLPQLGSGPTTDNFGSGYFSKADYVEILKYAKARNIEVI    441
CHB_SERMA     QRCHDLSETTCLLPQYGQGP--DVYG-GFFSRQDYIDIIKYAQARQIEVI    444
HEX_VIBVU     HRCHDVEQNKCMMPQLGSGAELPNNGSGYYTREDYKEILAYASARNIQVI    424
HEXB_ALTSO    YRCFDLTETRCLLPQLGAGNDKNAQVNGFYSAEDYIEILRYAKAHHIEVL    436
HEXA_PORGI    TR--------------TEGDGTQY--SGFYTQEQVRDIVQYASDHFITVI    263
                                                      .       .

HEXA_HUMAN    AEFDTPGHT-LS-----------WGPGIPG------LLTPCYS-GSEPSG    285
HEXA_MOUSE    AEFDTPGHT-LS-----------WGPGAPG------LLTPCYS-GSHLSG    285
HEXB_HUMAN    PEFDTPGHT-LS-----------WGKGQKD------LLTPCYS-RQNKLD    317
HEXB_MOUSE    PGFDTPGHT-QS-----------WGKGQKN------LLTPCYN-QKTKTQ    296
HEXB_FELCA    PEFDSPGHT-QS-----------WGKGQKD------LLTPCYN-EHKQSG    259
HEXA_DICDI    PEFDIPGHA-AA-----------WGIGYPE------LVATCPDYAANV--    270
HEX1_ENTHI    GEIDTPAHT-AS-----------WNLGYPG------VVANCWDYIVSTSM    259
HEX1_CANAL    PEIDMPGHARAG-----------WKQVDPT------IVECADAFWTDAAV    284
HEXC_BOMMO    PEFDAPAHVGEG-----------WQDTGLT------VCFKAEP-WTKFCV    329
CHB_VIBHA     PEIDMPAHARAAVVSMEARYDRLMEEGKEAEANEYRLMDPQDTSNVTTVQ    491
CHB_SERMA     PEIDMPAHA-ARVVSMEARYKKLHAAGKEQEANEFRLVDPTDTSNTTSVQ    493
HEX_VIBVU     PSMDMPGHSLAAVKSMEARYRKFMAEGDVVKAEMYLLSDPNDTTQYYSIQ    474
HEXB_ALTSO    PSLDMPGHSRAAIIAMEARYKKLMAQGKPEDAQKYRLVETADKTRYSSIQ    486
HEXA_PORGI    PMIEMPGHAMAALAA----YPQFRCFPREFKPRIIWGVE-----------    298
                 ■ . .

HEXA_HUMAN    TFG-----PVNPSLNNTYEFMSTFFLEVSSVF-----PDFYLHLGGDEV-    324
HEXA_MOUSE    TFG-----PVNPSLNSTYDFMSTLFLEISSVF-----PDFYLHLGGDEV-    324
HEXB_HUMAN    SFG-----PINPTLNTTYSFLTTFFKEISEVF-----PDQFIHLGGDEV-    356
HEXB_MOUSE    VFG-----PVDPTVNTTYAFFNTFFKEISSVF-----PDQFIHLGGDEV-    335
HEXB_FELCA    TFG-----PINPILNSTYNFLSQFFKEVSMVF-----PDHFVHLGGDEV-    298
HEXA_DICDI    ----NN-IPLDISNPATFTFIQNLFTEIAPLF-----IDNYFHTGGDEL-    309
HEX1_ENTHI    RYGENV-LSLNPANPNTFPIIDALMKELSDTF-----GTDYVHVGGDEV-    302
HEX1_CANAL    E--PPP-GQLNIESEKTYEVISNVYNELSDIF-----IDDVFHVGNDEL-    325
HEXC_BOMMO    E--PPC-GQLNPTKEELYDYLEDIYVEMAEAFE----STDMFHMGGDEV-    371
CHB_VIBHA     FYNKQSFINPCM--ESSTRFVDKVISEVAAMHQEAGAPLTTWHFGGDEAK    539
CHB_SERMA     FFNRQSYLNPCL--DSSQRFVDKVIGEIAQMHKEAGQPIKTWHFGGDEAK    542
HEX_VIBVU     HYQDNT-INPCM--ESSFVFMDKVIDEINKLHKEGGQPLTDYHIGADETA    521
HEXB_ALTSO    HYNDNT-LNVCI--ANTYTFIDKVLSEVKVLHDRAGVPLNTYHIGADETA    533
HEXA_PORGI    ---QDVY---CAGKDSVFRFISDVIDEVAPLF-----PGTYFHIGGDECP    337
                                        *               . . .*
```

b

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HEXA_HUMAN    IGGEACMWGEYV-DNTNLVPRLWPRAGAVAERLWS---------------    486
HEXA_MOUSE    IGGEACMWGEYV-DSTNLVPRLWPRAGAVAERLWS---------------    485
HEXB_HUMAN    IGGEACLWGEYV-DATNLTPRLWPRASAVGERLWS---------------    515
HEXB_MOUSE    IGGEACLWGEFV-DATNLDSKIMPRASAVGERLWS---------------    494
HEXB_FELCA    IGGEACLWGEFV-DATNLTPRLWPRASAVGERLWS---------------    457
HEXA_DICDI    IGGEATMWAEQI-NQVNWDVRVWPRAIGIAERLWS---------------    469
HEX1_ENTHI    LGGEGCSWGEST-DEQNFFDRVFQRYSAIAERLWS---------------    475
HEX1_CANAL    LGREAALWSEQV-DSTVLTTKIWPRTAALAELTWS---------------    506
HEXC_BOMMO    LGGEVALWSEQS-DPATLDGRLWPRAAAFAERMWA---------------    552
CHB_VIBHA     YGLSAQLWSETVRNDEQYEYMVFPRVLAAAQRAWH---------------    763
CHB_SERMA     YGLSAQLWSETQRTDPQMEYMIFPRALSVAERAWH---------------    763
HEX_VIBVU     LGVQGALWSETIRTDAQVEYMVLPRMIAVAERGWH---------------    726
HEXB_ALTSO    AGLQGHLWSEMLRSDAQAEYMLYPRLLALAERAWH---------------    727
HEXA_PORGI    LGAQANLWAEYLYTSERYDYQAYPRLLAVAELTWT---------------    503
               .     . *              .        .
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FIG. 4. **Multiple alignment of two regions of family 20 glycosyl hydrolases (*a* and *b*).** *Asterisks* (*) indicate invariant glutamate residues Glu-α307, Glu-α323, and Glu-α424. Residues at which B1 mutations occur (Arg-α178 and Asp-α258) are marked by (■). Other invariant residues are also indicated (·).

ticipate in substrate cleavage. In this study, we attempted to identify acidic residues in the Hex A α subunit which may participate in the active site of Hex A.

Since our studies involved the use of a novel TSD-NG expression system, we first evaluated expression in this system of wild type and mutant *HEXA* cDNAs. Transient expression of mutant *HEXA* cDNAs in COS1 cells (reviewed in Brown and

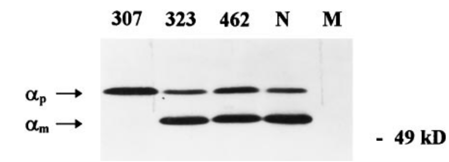

FIG. 5. **Western blot analysis of TSD-NG cells transfected with pCMVαE307D (*307*), pCMVαE323D (*323*), pCMVαE462D (*462*), and pCMVα (*N*).** *Lane M* represents mock-transfected cells.

Mahuran (34)) has several disadvantages which are overcome in the TSD-NG expression system. COS1 cells exhibit a high level of endogenous Hex A and Hex B activity which compromises the analysis of low levels of Hex activity expressed by mutant *HEXA* cDNAs. Furthermore, because the cells are of heterologous origin, human *HEXA* gene mutations are expressed in COS1 cells as the non-physiological isozyme Hex S (7, 37, 38), an αα homodimer which differs from Hex A both in stability and substrate specificity. As a result, the activity of mutant α subunits produced following transfection of mutant *HEXA* cDNAs, does not correlate well with clinical severity of disease or biochemical phenotypes observed in patients (34, 35).

In contrast, TSD-NG cells, produce no endogenous α subunits. Endogenous expression of *human* β subunits in these cells, however, permits dimerization with transfected human α subunits, to form active Hex A. When wild type α subunits are overexpressed in TSD-NG cells, β subunit concentration limits the formation of Hex A. As the β subunits become depleted, excess α subunits dimerize to form Hex S, distinguishable from Hex A by chromatofocusing. The presence of both the precursor (67 kDa) and mature (54 kDa) forms of α subunit following transfection confirms that newly synthesized α subunit can be normally processed and targeted to the lysosome, but also indicates that excess α subunits are poorly processed in the absence of β subunits.

We evaluated the expression in TSD-NG cells of α subunits carrying mutations associated with acute (αR170W), subacute (αG250D), and chronic (αG269S) forms of GM_2 gangliosidosis, respectively, and demonstrated that residual enzyme activity was inversely correlated with disease severity. Results for the αR170W mutation confirmed our previous report (11), that this mutation causes the classical infantile form of TSD. We proceeded to use this expression system to evaluate the role of candidate active site residues.

The four acidic amino acids we examined were chosen on the basis of (*a*) occurrence in the B1 variant of Tay-Sachs disease (Asp-α258) or (*b*) conservation in family 20, a sequence related family of glycosyl hydrolases (α307, α323, and α462). The TSD-B1 phenotype is believed to be caused by mutations which lead to substitution of residues with catalytic function (4, 7, 10). These include the basic residue Arg-α178 (4, 8–10) (which is invariant in family 20 enzymes) and the acidic Asp-α258 (7, 10). Conservative substitutions at these positions have little affect on K_m but dramatically reduce the V_{max} of Hex A (7). The residues Glu-α307, Glu-α323, and Glu-α462 were selected on the basis of their evolutionary invariance in family 20 glycosyl hydrolases (39), a classification system (29) based on amino acid sequence similarity. Members of homology families generally share folding characteristics and catalytic residues within families are strictly conserved (40). The three glutamate residues are invariant at homologous positions in all members of family 20. The position corresponding to Asp-α258 shows variation in a single enzyme which carries the functionally related acidic residue glutamate at this position.

Analysis of residual enzymatic activity and α subunit maturation in cells transfected with *HEXA* cDNAs carrying conserv-

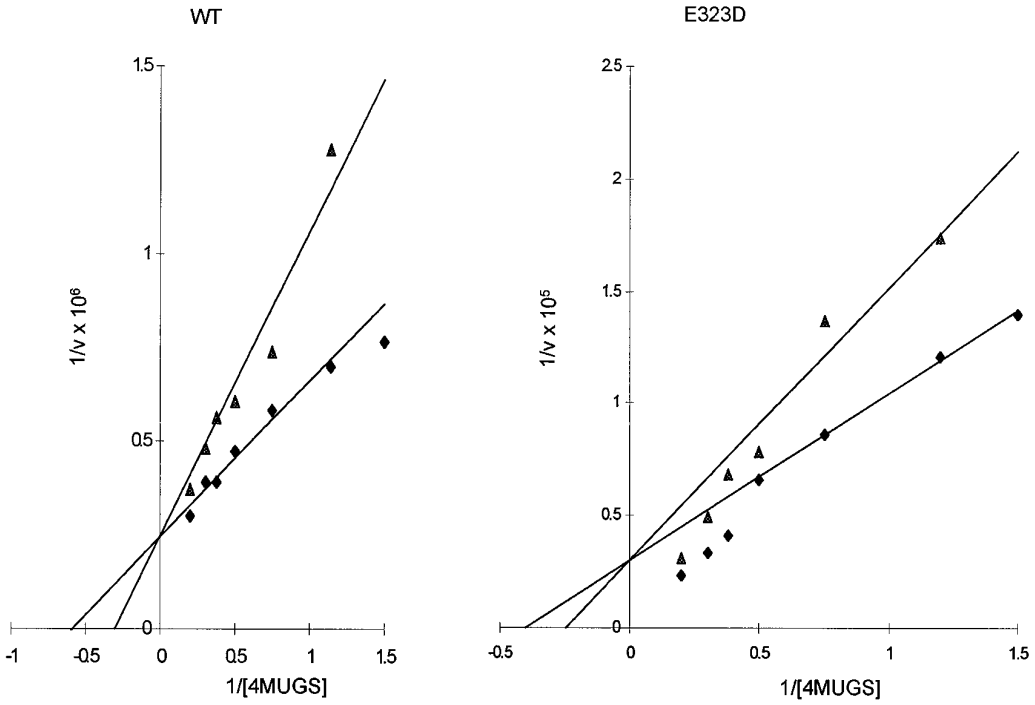

FIG. 6. **Lineweaver-Burk plots of wild type and αE323D mutant Hex A for hydrolysis of 4MUGS in the absence (♦), or presence (▲) of 10 mM *N*-aetylglucosamine-6-phosphate.** K_m for wild type Hex A = 1.7 mM. K_m for αE323D Hex A = 2.5 mM. K_i for wild type Hex A = 11.0. K_i for αE323D Hex A = 15.6 mM.

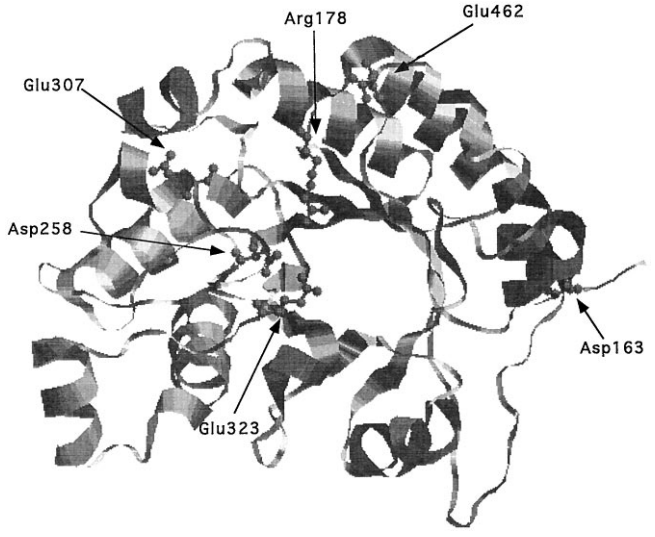

FIG. 7. **Predicted structure of the $(\beta\alpha)_8$ of hexosaminidase showing the apposition of residues Arg-α178, Asp-α258, and Glu-α323 in the active site pocket.**

ative or isosteric substitutions at residues Glu-α307 and Glu-α462 led us to conclude that these residues are unlikely to have catalytic functions. Residual Hex A activity associated with both mutations was too high to be compatible with the loss of either an acid catalyst or a proton donor. Furthermore, the αE307D substitution prevented α subunit maturation (Fig. 5). In contrast, both the isosteric substitution αD258N and the conservative substitution αE323D were associated with production of mature Hex A with drastically reduced enzyme activity. Since maturation of the α subunit is dependent on dimerization with the β subunit, we were also able to conclude that the αD258H and αE323D mutations are compatible with production of normal amounts of αβ heterodimer and that loss of activity is not the result of compromised dimerization. Furthermore, kinetic analysis of residual Hex A activity in αE323D transfected cells (Fig. 6) or αD258H in patient fibroblasts (7) demonstrates that loss of enzymatic activity is the consequence of decrease in k_{cat} rather than a change in K_m.

Our proposal that Asp-α258 and Glu-α323 are catalytic residues is in accord with results of other structure/function studies. The x-ray crystallographic structure for *Serratia marcescens* chitobiase, the first member of family 20 glycosyl hydrolases to be crystallized, was recently reported (26, 40, 42). Residues Glu-519 and Glu-740 of chitobiase are not located at or near the active site of the bacterial enzyme suggesting, by analogy, that the homologous residues, Glu-α307 and Glu-α462, in the α subunit of Hex A would also not be present at the the α subunit active site. Most significantly, the x-ray crystallographic data (26) identify Glu-540, the residue corresponding to Glu-α323, as the acid catalyst in the *S. marcescens* enzyme.

Members of family 20 glycosyl hydrolases share a conserved central region which aligns in all members of the family (Fig. 4). According to the crystal structure of chitobiase, this region comprises the catalytic domain which has an α/β barrel fold (26). The modeling of the catalytic domain of Hex A (Fig. 7) (26), brings Arg-α178, Asp-α258, and Glu-α323 in proximity within the substrate binding cleft (facing toward the center of the α/β barrel) and spatially arranged to facilitate catalysis. In contrast, Glu-α307, Glu-α462, and Asp-α163 which corresponds to Asp-β196, a residue recently proposed as an active site residue in the β subunit (44), appear to be remote from the catalytic domain. While it is likely that the model, illustrated in Fig. 7, will contain some errors in its proposed structure of Hex A, the high degree of conservation of residues in and around the active site suggest that the model is much less prone to errors in the active site pocket of the enzyme.

A role for Glu-α323 at the Hex A active site is also supported by the studies of Liessem *et al.* (43). Using a mechanism-based pyrrolidine substrate analog, this group identified residue Glu-β355, which corresponds to Glu-α323, as the only reactive residue at or near the β subunit active site. The role of Arg-α178 is less clear. Given the high pK_a of arginine (12.0) it is unlikely that this residue functions as a nucleophile at the pH

optimum of Hex (3.9–4.2). The role of active-site arginine may be to maintain the acid catalyst in its protonated state at a pH which is significantly higher than the pK_a for dicarboxylic amino acids.

The extensive sequence similarity of the α and β subunits underscores the structural and catalytic similarity of two subunits of Hex A. Chimeric Hex enzymes, generated by the fusion of different segments of the α and β subunits have recently been used to identify domains required for substrate specificity (41, 45). The structural modelling of Hex should now permit the more precise localization of other residues involved in the active site as well as in other functions of the enzyme, such as dimerization, substrate binding and activator recognition.

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