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

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Maria J. G. Fernandes‡§, Sandy Yew‡, Daniel Leclerc‡, Bernard Henriassat†,
Constantin E. Vorgias‡‡, Roy A. Gravel‡‡‡, Peter Hechtman§§§, and Feige Kaplan‡¶¶¶

From ‡McGill University-Montreal Children’s Hospital Research Institute, Departments of ‡Biologische, ‡Human Genetics, ‡‡Pediatrics, McGill University, Montreal, Canada H3H 1P3; ‡§Centre de Recherches sur les Macromolecules Vegetales, CNRS, Grenoble F-38041, Cedex 9, France; and ‡¶¶¶ European Molecular Biology Laboratory (EMBL), 22603, Hamburg, Germany

The β-hexosaminidases (Hex) catalyze the cleavage of terminal amino sugars on a broad spectrum of glycoconjugates. The major Hex isoforms 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 GM₂ ganglioside. Mutations in the HEXA gene cause Tay-Sachs disease (TSD), a GM₂ ganglioside storage disorder. We investigated the role of putative active site residues Asp-258, Glu-307, Glu-323, and Glu-462 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-307, Glu-323, and Glu-462 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-323 and Asp-258 (reported earlier by Baylerian, 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-307 and Asp-258 are active site residues and that Glu-323 is involved in catalysis.

The β-hexosaminidases (Hex, EC 3.2.1.52) are lysosomal hydrolases that catalyze the cleavage of terminal β-N-acetylgalcosamine or β-N-acetylgalactosamine residues on a broad spectrum of glycoconjugates. The major Hex isoforms 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 isoform, 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-SO₄) or on a distinct residue (e.g. sialic acid) (4). The latter includes the most important natural substrate, the sialic-acid containing ganglioside GM₁₄, found mainly in neuronal tissue. Only Hex A catalyzes cleavage of the terminal β-N-acetylgalactosamine on GM₂ ganglioside in the presence of the substrate-specific protein cofactor, the GM₂-activator protein (5).

Mutations in the HEXA, HEXB, and GMZA genes, encoding the α and β subunits of Hex A and the GM₂-activator, respectively, lead to a group of inherited neurodegenerative diseases, collectively known as the GM₂ gangliosidoses, that are characterized by lysosomal accumulation of GM₂ 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 GM₂ 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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‡‡ On leave at Athens University.

¶¶¶ To whom correspondence should be addressed: McGill University-Montreal, Children’s Hospital Research Institute, 2300 Tupper St., Montreal, PQ Canada H3H 1P3. Tel.: 514-934-4417; Fax: 514-934-4329.

¶¶¶¶ The abbreviations used are: Hex, hexosaminidase; TSD, Tay-Sachs disease; HEXA, HEXB, and GMZA, the genes for the α and β subunits of Hex A and the GM₂ ganglioside activator protein, respectively; AMU, 4-methylumbelliferone; 4MUGS, 4-methylumbelliferone β-N-acetylgalcosamine; 4MUGS, 4-methylumbelliferone N-acetylgalactosamine-6-sulfate; NG, neuroglial; PCR, polymerase chain reaction.

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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 chitinas (24, 25) and the β-N-acetylgalactosaminidase (chitobiase) (26). In contrast, based on inhibition studies using nitrogen-containing substrate inhibitors, Legler and Boothagen (27) and Legler (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 neural cell (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—NG1141 (NG) and NG125 (TSD-NG), SV40-transformed fetal TSD and normal neural 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 pSVLgal (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 pCRRI (Invitrogen) as an XbaI/BamHI fragment, 2) subcloning of an NsiI HEXA cDNA-containing fragment PCRII subclone into the NotI site of pBluescript (Stratagene), 3) subcloning an XhoI/XbaI fragment from pBluescript into a PCRII intermediate, and 4) cloning a NotI/XhoI HEXA cDNA-containing fragment from the PCRII subclone into PRCCMV. Preparation of pREP4α involved cloning the XhoI/BamHI HEXA cDNA-containing fragment from PSVLα into pREP4. Construction of pCMVα required creating a HEXA cDNA flanked by NotI sites. The HEXA cDNA insert in pRCCMVα has a NotI site at the 5’ end of the gene. This insert was subcloned into the BamHI site of pBluescript (KS–, Stratagene) introducing a second NotI site downstream from the 3’ end of the gene. This step permitted the cloning of the insert into the unique NotI 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 NG125, subconfluent T175s (175 cm², Sarstedt) were harvested by trypsinization and washed in MEM medium with 1 x 10⁶ cells/ml. The cells were resuspended in OptiMEM (Life Technologies, Inc., containing 5% fetal bovine serum) to obtain a final concentration of 6 x 10⁶ cells/ml. Cell suspension (800 μl), 20 μg of pCMVα, and 2 μg of pSVLgal 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 electroporator. 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 multwell plates. The remainder of the transfected cell suspension was grown for 48 h in a T75 (75 cm²).

Enzyme and Protein Assays—A qualitative β-galactosidase assay was performed to determine the percentage of surviving cells which expressed bacterial enzyme. Multwell-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 x magnesium solution (4.5 M 2-mercaptoethanol, 0.1 M MgCl₂), 100 μl of 0.5 mM 4 methyl-umbel liferyl-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 μl. 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 antibody were horseradish peroxidase-labeled (anti-rabbit) antibodies were used at a 1:5000 dilution.

Chromatofocusing of Transfected Cell Extracts—The hexosaminidase isozyme profile in cell lysates was determined by chromatofocusing using the Pharmacia Polybuffer Exchanger (PBE) 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 BPE74 slurry was used to prepare a column in a 1-ml syringe. The column was washed with the equivalent of 1.5–2 x bed volume with 0.025 M imidazole buffer. The protein extract was added after passing 2 x 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 Km and Kf 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-acetylgalactosamine-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 x mock-trans-
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 a 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. 2a 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) subacuate (αG250D) (35) and chronic (αG269S) (37) forms of GM2 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 αD258E, αD258N, or αD258S 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 hydrolisys by Hex A and Hex S (data not shown). The 4MUGS hydrolisys of Hex A was reduced to a lesser extent. This result was expected since 4MUGS hydrolisys 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-

### Table I

| Vector          | Time harvested | Hex activity |
|-----------------|----------------|--------------|
|                 | h             | nmol/h/mg    |
| Untransfected NG cells |              | 715 (±13)    |
| Untransfected TSD-NG cells |              | 3 (±2)       |
| pSVLα          | 48            | 43 (±15)     |
| PCMVα         | 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). αp and αm indicate precursor and mature forms of Hex A α subunit. Lane M represents mock-transfected cells.](image-url)
ern blot analysis of transfected cell extracts showed that only the aE323D and aE462D mutations were compatible with synthesis of significant amounts of precursor and mature a subunit (Fig. 5). Chromatofocusing of aE323D and aE462D cell extracts confirmed that the Hex A isoenzyme is processed normally but has a catalytically defective a subunit that is unable to hydrolyze 4MUG or 4MUGS (Fig. 2, b and c). The aE307D mutation, however, dramatically reduces a subunit maturation.

Kinetic experiments were performed to evaluate the impact of the aE323D mutation on the binding affinity of Hex A for the substrates 4MUGS and for the competitive inhibitor N-acetyl-glucosamine-6-PO4 (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 aE323D enzyme are biphasic revealing a significant contribution of a second active site (the b subunit) at high substrate concentrations. When the kinetic parameters of the high affinity site are evaluated the Km for 4MUGS is 2.5 mM for Hex A aE323D compared to 1.7 mM for the wild type enzyme. Similarly Ki values for the competitive inhibitor are 15.6 mM for the aE323D-substituted Hex A and 11.0 mM for wild type Hex A. We showed, previously, that the Km 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).

**FIG. 2.** Chromatofocusing of NG cell extracts transfected with pCMVα, pCMVαE323D, and pCMVαE462D and assayed with the synthetic substrates 4MUG (■) and 4MUGS (□ — □). Activity peaks for Hex B, Hex A, and Hex S are indicated. (——) pH gradient.

**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.
Mutant HEXA cDNAs are also indicated (Fig. 4). Asterisks (*) indicate invariant glutamate residues (462), which may affect on K_m of Hex A (7). The four acidic amino acids we examined were chosen on the basis of (a) occurrence in the B1 variant of Tay-Sachs disease (Fig. a) and (b) conservation in family 20, a sequence related family of glycosyl hydrolases (307), (332), and (346). The TSD-B1 phenotype is believed to be caused by mutations which lead to substitution of residues with catalytic function (4, 7, 10). Conservative substitutions at these positions have little effect on Km of Hex A, but dramatically reduce the V_max of Hex A (7). The residues Glu-a307, Glu-a323, and Glu-a462 were selected on the basis of their evolutionary invariance in family 20 glycosyl hydrolases (39), a classification system (29) based on amino acid sequences 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 enzymes and the acidic Asp-a258 (7, 10) (which is invariant in family 20 glycosyl hydrolases). We evaluated the expression in TSD-NG cells of a subunit carrying mutations associated with acute (a178), subacute (a307, a323, and a462) and chronic (a323) disease or biochemical phenotypes observed in patients (34, 35).

In contrast, TSD-NG cells, produce no endogenous a subunits. Endogenous expression of human β subunits in these cells, however, permits dimerization with transfected human a subunits, to form active Hex A. When wild type a subunits are overexpressed in TSD-NG cells, β subunit concentration limits the formation of Hex A. As the β subunits become depleted, excess a 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 a subunit following transfection confirms that newly synthesized a subunit can be normally processed and targeted to the lysosome, but also indicates that excess a subunits are poorly processed in the absence of β subunits.

The four acidic amino acids we examined were chosen on the basis of (a) occurrence in the B1 variant of Tay-Sachs disease (Asp-a258) or (b) conservation in family 20, a sequence related family of glycosyl hydrolases (a307, a323, and a462). 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-a178 (4, 8–10) (which is invariant in family 20 enzymes) and the acidic Asp-a258 (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-a307, Glu-a323, and Glu-a462 were selected on the basis of their evolutionary invariance in family 20 glycosyl hydrolases, a classification system (29) based on amino acid sequences 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-a258 shows variation in a single enzyme which carries the functionally related acidic residue glutamate at this position.
ative or isosteric substitutions at residues Glu-a307 and Glu-a462 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 aE307D substitution prevented a subunit maturation (Fig. 5).

In contrast, both the isosteric substitution D258N and the conservative substitution E323D were associated with production of mature Hex A with drastically reduced enzyme activity. Since maturation of the a subunit is dependent on dimerization with the b subunit, we were also able to conclude that the d258N and aE323D mutations are compatible with production of normal amounts of a/b heterodimer and that loss of activity is not the result of compromised dimerization. Furthermore, kinetic analysis of residual Hex A activity in aE323D transfected cells (Fig. 6) or d258N in patient fibroblasts (7) demonstrates that loss of enzymatic activity is the consequence of decrease in $k_{\text{cat}}$ rather than a change in $K_m$.

Our proposal that Asp-a258 and Glu-a323 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-a307 and Glu-a462, in the a subunit of Hex A would also not be present at the a subunit active site. Most significantly, the x-ray crystallographic data (26) identify Glu-540, the residue corresponding to Glu-a323, 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 $a/b$ barrel fold (26). The modeling of the catalytic domain of Hex A (Fig. 7) (26), brings Arg-a178, Asp-a258, and Glu-a323 in proximity within the substrate binding cleft (facing toward the center of the $a/b$ barrel) and spatially arranged to facilitate catalysis. In contrast, Glu-a307, Glu-a462, and Asp-a163 which corresponds to Asp-\beta196, a residue recently proposed as an active site residue in the $b$ 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-a323 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-\beta355, which corresponds to Glu-a323, as the only reactive residue at or near the $b$ subunit active site. The role of Arg-a178 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 pKₐ 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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