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Identification of two amino acid residues which determine the substrate specificity of human β-D-N-acetylhexosaminidase A

β-D-N-acetylhexosaminidases A (Hex A, αβ) and B (Hex B, ββ) cleave N-acetylglucosamine and N-acetylgalactosamine termini of glycoconjugates. Hex B hydrolyzes neutral substrates whereas Hex A also hydrolyzes electronegative substrates, including GM_2 ganglioside, which accumulates in the neurons of patients with Tay-Sachs disease (TSD).

We hypothesized that enzyme-substrate electrostatic interactions influence substrate specificities of the two isozymes. Among seven positively charged candidate residues in Hex A, at which substitution for the homolgous β residue was performed, only the α424R→L mutation resulted in loss of activity toward the electronegative substrate 4-methylumbellifery-N-acetylglucosamine-6-sulfate (4MUGS). The substitution L→R at the homolgous β position 453 increased Hex B activity to 4MUGS 5-fold.

αR453 projects into the α-subunit substrate cavity opposite three active site amino acids. The adjacent residue, βD452, may repel negatively charged substrates. Double substitution, βL453→R and βD452→N (the α-subunit homologue), increases 4MUGS hydrolysis by 22-fold relative to wild type Hex B.

These results indicate that the homology model for hexosaminidase gives an accurate picture of the active site region and may furnish other candidate residues to test as determinants of the unique substrate specificity of Hex A.

Keywords: Tay–Sachs disease, GM_2 gangliosidosis, hexosaminidase, enzyme active site

1 Introduction

The β-hexosaminidases (Hex[1], E.C. 3.2.1.52) are lysosomal hydrolases which 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 composed of two β subunits. A third isozyme, Hex S, which is composed of two α subunits, is unstable and not normally found in most tissues. The α and β subunits are structurally related, sharing 54% 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 both neutral and charged substrates. Only Hex A catalyzes cleavage of GM_2 ganglioside, the most important natural substrate, in the presence of a substrate-specific cofactor, the GM_2 activator protein [4].

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Mutations in the HEXA gene produce deficient or defective α-subunits which are clinically associated with Tay-Sachs Disease (TSD, OMIN 272800), an infantile neuro-degenerative disorder caused by massive accumulation of the sialylated glycolipid GM_2 ganglioside in the neurons of the cerebral cortex. The presence of normal levels of Hex B in TSD patients indicates that the ganglioside substrate is not cleaved by this enzyme. In contrast, mutations in the *HEXB* gene produce deficient or defective β-subunits and are clinically associated with Sandhoff Disease (OMIN 268800). Patients with this disorder lack both, Hex A and Hex B, and accumulate, in addition to GM_2 ganglioside, a variety of other acidic and neutral glycoconjugates [5].

Differences in substrate specificity between the hexosaminidase isozymes have been confirmed by investigations of the properties of the purified enzymes. The biochemical basis for the differences in substrate specificity is complex. Hydrolysis of GM_2 ganglioside by Hex A requires that the ganglioside substrate form a complex with the GM_2 activator protein prior to cleavage by the enzyme. The activator, which is the product of the *GM2A* gene, facilitates hydrolysis of GM_2 ganglioside by binding to ganglioside monomers and releasing them from membranes or micelles. Hex B does not recognize the activator-substrate complex [6, 7, 4]. A second important difference in substrate specificity between Hex A and Hex B is that while the active site of both enzymes hy-

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drolyzes neutral substrates, only the α-subunit active site, present in Hex A and Hex S, but not in Hex B, hydrolyzes negatively charged substrates [8, 9, 3, 10].

Early studies of the catalytic mechanism of hexosaminidase enzymes focused on the identification of amino acid residues comprising the enzyme active sites. Three Hex A residues, R178 [11], D258 [12], and E323 [13] were shown to be essential for catalysis. Experiments using site-directed mutagenesis to introduce conservative substitutions at each of these positions caused loss of capacity of the mutant enzyme to hydrolyze both neutral substrates, such as the synthetic substrate 4MUG and electronegative substrates, such as 4MUGS [11]. Moreover, investigation of the active site residues of Hex B revealed that the same three residues perform a catalytic role in this enzyme. E355 in the β subunit, which is homologous to aE323, is the only residue that is labeled when Hex B is incubated with the photo-affinity suicide substrate [^{3}H]-1-ATB-N-acetylgalactosamine [14]. Similarly, replacement of R211, the β-subunit homologue of αR178, by a lysine residue leads to loss of activity in the mutant Hex B. The residue at position α278, the β-subunit homologue of α258, has not been evaluated using site-directed mutagenesis experiments, however, an aspartate is found at both positions. The finding that the two subunits share a common set of active-site residues indicated that differences at other codon positions must account for their substrate-specificity differences.

Pennybacker et al. [15] and Tse et al. [16] constructed and expressed chimeric Hex subunits containing α- and β-subunit sequences. Both investigators found that selected α-subunit sequences substituted into the β-subunit cDNA sequence confer on the chimeric protein partial activity toward a-subunit specific substrates. However, the two groups did not agree on the α-subunit sequences that were essential.

We are investigating the structural basis of the Hex A α-subunit substrate specificity. We hypothesized that since the α and β subunits are 54% identical (64% homologous) with respect to amino-acid sequence, differences in substrate specificity would be determined by primary sequence differences occurring at the 1/3 of the residues that are not similar in the two subunits. Since substrate specificity differences between the α and β active sites involve the negative charge on the substrate, we hypothesized that residues which are critical for the catalytic specificity of the isozymes are likely to be those which are electrically charged and not matched by identical or chemically similar residues at corresponding positions in the two chains.

In this report, we describe the identification of two amino acid residues that contribute to the catalytic specificity of the α and β chains of the hexosaminidases. These residues have been implicated in catalytic specificity by site-directed mutagenesis experiments and studies of the substrate specificity of the expressed mutant hexosaminidase constructs. The putative role played by each of these two amino acids is further supported by modeling studies which demonstrate that both of the candidate residues project into the substrate cavity which also contains the active site residues of the hexosaminidase isozymes.

2 Materials and Methods

2.1 Plasmid

pCMV-β-gal, containing the *E. coli* β-galactosidase gene, was purchased from Clontech (Palo Alto, CA, USA). The recombinant plasmid pCMVα, containing the wild-type human HEXA cDNA was prepared as described in [13]. pCMVβ, containing the wild-type human HEXB cDNA was prepared from the plasmid pHexβ43 [17], obtained from D. Mahuran and R. Gravel (Toronto Sick Children's Hospital). The *HEXB* insert was PCR amplified using the primers HxB-1 (5′-TTGCGGCCGCTTTATG CTGCTGGCGCTGCTGTTGG-3′) and Hx B2-2 (5′-CGTGCGGCCGCTTACATGTT CTCATGGTTACAATATCC-3′), both of which contain Not 1 linkers. Amplification was performed with Hot Start for 2 min at 94 °C followed by 1 min at 94 °C, 1 min at 50 °C, 3 min at 72 °C, for 30 cycles followed by 7 min at 72 °C. The reaction was catalyzed by Platinum TAQ HiFi from Gibco/BRL address. The PCR fragment was digested with Not 1. A linearized pCMV fragment was created by digesting the pCMVα plasmid with Not 1 and the plasmid fragment was gene-cleaned and dephosphorylated. The linearized pCMV fragment was ligated to the Not 1-digested PCR amplified HEXB cDNA using T4 DNA ligase (Gibco/BRL). The orientation of the HEXB insert within the plasmid was verified by the appearance of a 550bp fragment following double digestion with EcoRV and SacI.

2.2 Mutagenesis

Mutations were introduced into the pCMVα and pCMVβ plasmids using a Clontech mutagenesis kit. In all pCMVα mutagenic reactions the selection primer was CGACGGCCAGTGATATCGAGCTTGC, where the underlined bases serve to replace the vector EcoR1 site with an EcoRV site. The following mutagenic primers were used to prepare mutant forms of *HEXA* and *HEX*B containing plasmids. The underlined base(s) indicate the replacements: αR69H, CCAGCGCTATCAATGACCTGCTTTTC; αK148Q, GCTTGTTTGGCAATCTGCTGAGGGC; αK158E, CTTATCAACGAGACTGAGATTGAGGACTTTCCCCGC; αK402E, GTGAACTATATGGAGGAGCTGGAACTGG; αK409A, GGAACTGGTCACCGCGGCCGGCTTCCG; αR413P, GGCCGGCTTCCTGGCCCTTCTCTCTG; αR424L, GGTACCTGAACCTTATATCCTATGGCCC. For mutagenesis of plasmid pCMVβ in which mutations were intro-

duced into codons 317, 452, and 453 the selection primer was GTGACTGGTGAGGCCTCAACCAAGTC which converts a ScaI site on the vector to an Stu1 site. Mutagenic primers were, βD317G, GACAAAACAAGTTGGCCTCTTTTGGACC; βD452N, CCTTGGGTACTTAAATTTGATTAGCTATGG; βL453R, GGTACTTAGATCGGATTAGCTATGG. The double mutant containing βD317G and βD452N were prepared using the same selection primer and both mutagenic primers described for the corresponding single mutations. The double mutant constructs containing βL453R as one of the mutations were prepared using the pCMVβ453L→R as template with a selection primer CTTCCTTTTTCGATATCATTGAAGCATTT which changes the Ssp1 site in the vector to an EcoR5 site and with each of the two mutagenic primers described above for codons 317 and 452. The triple mutation was created using pCMVβ453L→R as template, with the Ssp1→EcoR5 selection primer and the mutagenic primers for codon 317 above and CCTTGGTACTTAAATCGGATTAGCTATGG for mutagenesis at codon 452. The mutant cDNA sequences were verified by dideoxy sequencing [18]

2.3 Cell culture and transfection

NG125, an SV40 transformed neuroglial cell line obtained from a patient with Tay-Sachs disease, was provided by L. Hoffman and S. Brooks (Kingsbrook Jewish Medical Center, Brooklyn, NY) [19, 20]. This cell line does not express the *HEXA* gene, but does express the *HEXB* gene. Culture and transfection conditions were reported previously [13]. All transfections included pCMVβgal serving as a control for transfection efficiency.

2.4 Analytical procedures

β-galactosidase was measured according to Hearing et al. [21]. Hexosaminidases were measured with both 4MUG and 4MUGS (both from Sigma, St. Louis, MO, USA) substrates as previously described [8]. Chromatofocusing of cell extracts was performed according to [22]. Protein was determined by the method of Lowry [23].

2.5 Three dimensional mapping of amino acid residues

The Hex A β subunit 3D structure file was obtained from the Brookhaven Protein Data Bank (PDB code: 1QBD). The sequences of the β-hexosaminidase α and β chains were aligned according to Proia [2]. The location of each of eight negatively charged β-subunit residues, matched at corresponding α-subunit positions by neutral or positively charged residues, was examined using Rasmol 2.7.1© (Roger Sayle, 1992−1999; Herbert J. Bernstein, 1998−1999). Hydrophilicity of the region surrounding these residues (i.e. whether these residues extend into the catalytic pocket and are not buried) was verified using WinPep 2.0© (developed by Lars Hennig at the University of Freiburg, Germany and available from the Internet from http://www.biologie.uni-freiburg.de/data/schaefer/lhennig/winpep.html) by the method of Kyte and Doolittle [24].

3 Results

3.1 Residues exhibiting charge differences in the α and β subunits of Hex A

Seven positions were identified in the α-subunit polypeptide chain at which a positively charged residue is matched at the homologous position in the β-subunit by an electronegative or uncharged residue. The location of these sites and their β-chain homologues are indicated in Table 1.

Table 1. Site-directed mutagenesis of selected α-subunit residues and substrate specificity of mutant enzymes

Mutant No.	α-Subunit Residue	β-Subunit Residue	4MUG/4MUG
WT			0.36
1	Arg-69	His-102	0.34
2	Lys-148	Gln-181	0.36
3	Lys-158	Gln-191	0.37
4	Lys-402	Glu-431	0.32
5	Lys-409	Ala-438	0.35
6	Arg-413	Pro-442	0.37
7	Arg-424	Leu-453	<0.007

3.2 Expression and substrate specificity of α-subunit mutations

Mutant α-cDNAs were constructed in which the α-subunit residue at each of these positions was mutagenized to the corresponding β-subunit residue using site-directed mutagenesis in pCMV vectors. Mutant constructs were transfected into NG-125 cultured cells which, in the untransfected state, express only hexosaminidase β-subunits. In this transient expression system α subunits are over expressed in transfected cells (10-20% of cells) resulting in modest levels of Hex A expression and very high levels of Hex S expression. Hex B is expressed from the endogenous *HEXB* gene. The expressed isozymes were resolved by chromatofocusing of cell lysates. The results of a typical experiment are shown in Figure 1. The chromatofocusing profiles obtained for lysates of cell cultures transfected with six of the mutant *HEXA* constructs were virtually identical to the profile obtained, following expression of the wild type *HEXA* cDNA (Figure 1a and Table 1). By contrast, expression of the construct carrying the αR424L mutation re-

sulted in the chromatofocusing profile for the mutant enzyme shown in Figure 1b. Note that in this experiment greater volumes of all column fractions were assayed with both substrates in order to permit detection of low levels of activity toward 4MUGS. Despite this attempt to increase the sensitivity of the assay, no enzyme activity was detectable when 4MUGS was the substrate. The presence in the chromatofocusing eluate of enzymatic activities toward 4MUG at pIs corresponding to Hex A and Hex S indicated that the mutation did not prevent the expression of the α-cDNA and that normal processing of the α-subunit protein including transit through the internal membrane systems of the cell, folding, and dimerization occurred. The appearance of a peak of enzymatic activity with the 4MUG substrate at a pI corresponding to Hex S indicated that the catalytic site of the mutant a-chain is unaffected. However, the failure of the mutant Hex S to cleave the 4MUGS substrate indicated that the substrate specificity of this active site resembles more closely that of the β-subunit than the α-subunit. The substrate specificities of mutant and wild-type α subunits are indicated by the ratio of 4MUGS/4MUG activity for each Hex S peak in Table 1.

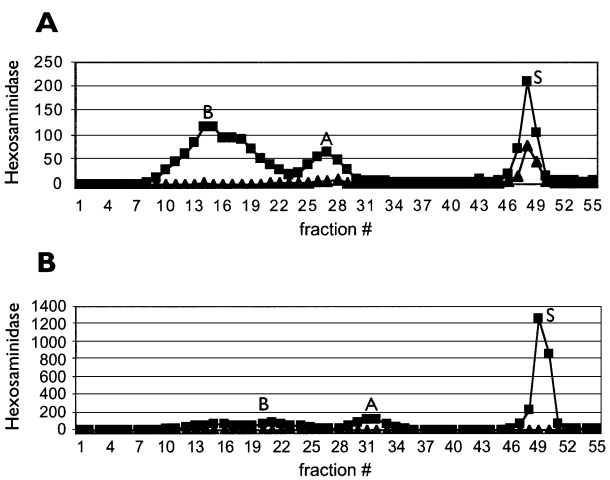

Figure 1. Separation of Hexosaminidase Isozymes by Chromatofocusing:
A. NG-125 Neuroglial cell line transfected with wild type pCMV Hex α. B. NG-125 Neuroglial cell line transfected with pCMVHexα-R424L. ■ Column fractions assayed with 4MUG. ▲ Column fractions assayed with 4MUGS

3.3 Expression of the βL453R mutation

The experiment described above indicated that α424Arg is necessary for hydrolysis of the electronegative substrate 4MUGS at the active site of the Hex A α-subunit. These results did not determine, however, whether this residue is sufficient to define the unique α-subunit substrate specificity. A reciprocal mutant construct was therefore prepared in which the leucine residue at the position 453 of the β-subunit (homologous to position 424 of the α-subunit) was converted to arginine. Following expression of this mutant β-subunit cDNA cells were harvested and cell lysates assayed for hydrolysis of 4MUG and 4MUGS.

Since the mutation did not significantly affect the pI of the expressed Hex B, the lysates were not chromatofocused prior to enzyme assays. Cells transfected with either wild type or mutant β-cDNAs had a specific activity for 4MUG hydrolysis between 3-4-fold greater than that of untransfected cells. These results indicated that the βL453R mutation did not prevent expression of the β-subunit nor interfere with its catalytic function. The ratio of 4MUGS/4MUG hydrolysis, however, was 8.5×10^{-3} for the mutant Hex B and only 1.6×10^{-3} for wild-type Hex B. Although the substitution of a single amino acid residue increased the ability of the β-subunit active site to hydrolyze 4MUGS by a factor of more than 5-fold, this increase in 4MUGS hydrolysis accounts for only about 2.4% of the activity of the a-subunit active site toward this substrate. The 4MUGS/4MUG ratio for wild-type Hex S was 0.36.

3.4 α424 localized to the substrate cavity of the Hex A catalytic dimer

The position of the α424Arg residue within the three dimensional structure of the α subunit was examined in order to determine whether the location of the arginine side chain was compatible with a role in neutralizing the charge on the electronegative substrate. A model structure for the Hex A α-subunit was proposed by Tews et al. [25] based on comparison with the X-ray diffraction measurements obtained on a crystallized enzyme-substrate complex of the related enzyme chitobiase. The model contains a central hydrophilic region referred to as the "substrate cavity". Figure 2 shows only this portion of the proposed structure. The three residues implicated in active site function cluster on the "left" side of the cavity. Arginine 424 projects into the substrate cavity from the opposite direction. Assuming that one of the three active-site amino acids interact with the glycosidic oxygen atom of the substrate, it is possible to compute the linear distance between the most distal H of Arginine 424 to the most distal atoms of the three active-site amino acids and compare these distances to the distance between the glycosidic oxygen of the substrate and the sulfur linked O atoms of the N-acetylglucosamine sulfate with which the arginine residue at position 424 might form an electrostatic bond. The distances between the active site amino acids and α-Arg424 range from 10.6−17 Å. Between the glycosidic O and the sulfur-linked O atoms of the substrate there are eight atomic bonds with an approximate distance of 1.5 Å, which would be equivalent to a linear distance of 12 Å. Since the bond angles are all less than 180°C the actual distance between the substrate negative charge and the site of enzymatic

cleavage is certainly less than the computed linear distance. This comparison, while very approximate, does indicate that the boundaries of the substrate cavity could accommodate an N-acetylglucosamine-6-sulfate moiety, permitting interaction of the substrate with both the Arg424 and the three active-site amino acids.

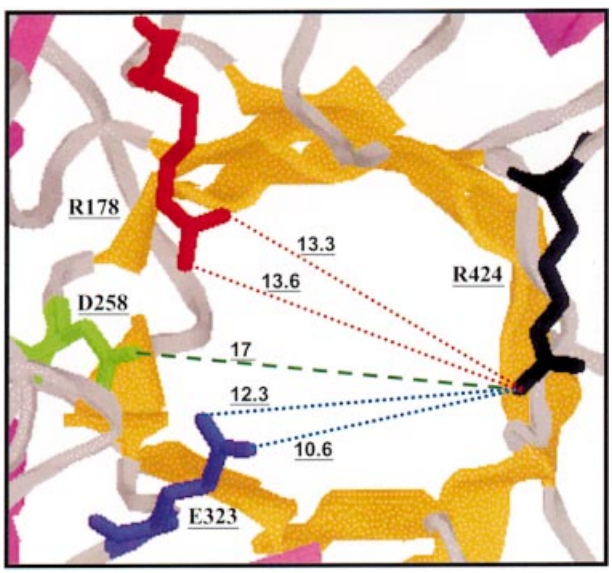

Figure 2. Substrate cavity of Hexosaminidase α-subunit: Numbers above dashed and dotted lines indicate distance in Å from guanidine-H atoms of Arg424 to most proximal atoms of the active site residues E323 (purple), D258 (green) and R178 (red)

3.5 A second Hex A-specific residue affecting substrate specificity

A corollary to our hypothesis is that negatively charged residues projecting into the substrate cavity of Hex B may serve to repel the approach of a negatively charged substrate into the active site. According to this prediction, neutral or positively charged residues at α-subunit positions that are matched by electronegative amino acids in the β subunit will influence the substrate specificity of the α-subunit active site. Table 2 shows a list of eight negatively charged residues at positions in the β subunit matched by neutral or positively charged residues in the corresponding α-subunit positions. We determined the position of each of these residues in the Hex B model structure with respect to the substrate cavity. Figure 3 shows the location of each of the eight electronegative residues listed in Table 2 on a version of the model that shows only the substrate cavity. Of the eight indicated residues only β317D and β452D appeared to be proximal to the substrate cavity and therefore likely to affect substrate specificity. The latter residue is one peptide bond away from β453L which is the β-subunit homologue of α424R.

The effect on the substrate specificity of Hex B of substitutions of each of these residues by their a-subunit homologues was next investigated. The mutant αDNAs prepared are indicated in Table 3. Each of the two substitutions, β317D→G and β452D→N, were introduced into the wild-type β-cDNA. In addition, mutations at positions β317 and β452 were introduced into a β-cDNA already carrying Arginine at position β453 (the β-subunit homologue of α424). Lastly, a triple mutant was constructed carrying the β317G, β452N, and β453R. Following transfection, lysates of harvested cells were assayed for activity to both substrates. Table 3 reports the results obtained.

Table 2. Candidate Hex B residues and their Hex A Homologues

HEX B	HEX A
Asp-304	Gly-272
Asp-317	Gly-285
Glu-342	Ser-310
Glu-362	Lys-330
Asp-411	Asn-379
Glu-431	Lys-402
Asp-452	Asn-423
Asp-470	Ala-441

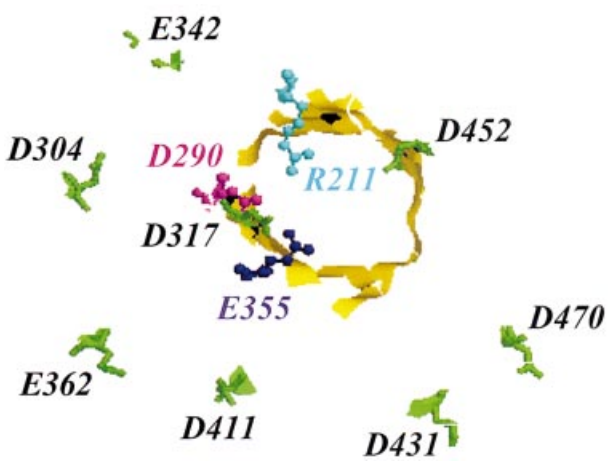

Figure 3. Substrate cavity of Hexosaminidase ß-subunit: Portion of peptide chain in yellow represents substrate cavity with active site residues, D290 (fuscia), R211 (turquoise) and E355 (purple). Residues in green are acidic residues in the ß-subunit that are matched in the corresponding α-subunit positions by non-acidic residues.

The Glycine residue at α285 appeared to have little or no effect on the ability of the β-subunit active site to hydrolyze 4MUGS when introduced at the homologous β317 position either in the β-cDNA carrying the wild-type Leucine residue at position β453 or the mutant Arginine residue. The Aspara-

Table 3. Substrate Specificity of Mutant Hex Bs

cDNA	β453	β317	β452	4MUGS/ 4MUG ($\times 10^{-3}$)	% Relative to HEX S
α		wild type		360	100
β(WT)	Leu	Asp	Asp	1.6	0.44
β	Leu	Gly	Asp	1.74	0.48
β	Leu	Asp	Asn	2.4	0.66
β	Leu	Gly	Asn	2.7	0.74
β	Arg	Asp	Asp	8.5	2.3
β	Arg	Gly	Asp	3.2	0.88
β	Arg	Asp	Asn	35	9.6
β	Arg	Gly	Asn	2.9	0.81

gine residue at α423 appeared to have only a modest effect on hydrolysis of 4MUGS when introduced into the corresponding β452 position in a β subunit having the wild-type Leucine at position 453. However, when an Arginine residue occupied position 453, the rate of 4MUGS hydrolysis by the double mutant was increased by a further 4-fold compared to the activity of the Hex B carrying the single substitution β453L→R. The double mutant construct thus encodes a form of Hex B in which the capacity for 4MUGS hydrolysis is 9.6% that of Hex S, an increase of about 20-fold in the capacity of this enzyme for hydrolysis of electronegative substrates. In experiments in which mutant forms of Hex B are expressed there is no separation of the mutant enzyme from the wild type endogenous product of the host cell genomic DNA. The latter enzyme typically accounts for 1/4 to 1/3 of the Hex B detected in cells harvested after transfection with β-cDNA. Allowing for the "contamination" by endogenous Hex B would increase the capacity of the Hex B encoded by the double mutant construct for 4MUGS hydrolysis to 12−13% of that of Hex S.

4 Discussion

Knowledge of the structural basis for substrate specificity is becoming available for an increasing list of enzymes. Experimentally, the requirements for identification of substrate-binding residues are: 1) Detailed kinetic studies of at least two enzymes catalyzing identical reactions but on different groups of substrates, 2) structural models for the enzymes permitting identification of the positions of residues that may contribute to a substrate-binding pocket, and 3) site-directed mutagenesis and expression of mutant enzymes to test hypotheses concerning the role of candidate residues.

X-ray diffraction is considered to be the "gold standard" of protein structural studies. Structures delineated by X-ray have been used to identify substrate-binding residues in a variety of enzymes, e.g. [26−32]. Recently, the number of protein structures that have been resolved has greatly increased due to the application of computerized homology modeling software. These structures have also been successfully used to predict substrate-binding residues, e.g. [33−37]. Structures based on homology modeling require verification by other types of experimental evidence.

The above studies lend support to the model for the α and β subunits of human hexosaminidase published by Tews et al. [25]. This model contains a central hydrophilic cavity which the authors designated as the "substrate cavity". We have previously reported [13] that the proposed structure was reinforced by the observation that three amino acids with proven catalytic function projected into the substrate cavity. We have extended the functional characterization of the substrate cavity by demonstrating a role for two additional amino acids found at this site in the determination of the substrate specificity of the enzyme. Sequence differences between the α and β subunits at these two positions can account for only 1/7 of the substrate-specificity difference. Although common sense considerations might dictate that residues affecting substrate specificity would project into this substrate cavity our initial search for such residues did not impose this requirement on the candidate residues selected for testing. The first residue identified, αArg424, was selected as a candidate based on 1) its positive charge and 2) the absence of a positively charged residue in the corresponding position in the β-subunit. Only subsequent to the functional characterization of this residue was its position examined. The linear distances between Arg424 and the most distal atoms of the three active site amino acids have been determined. These distances compare approximately to the length of the atomic bonds spanning the distances in glucosamine-6-sulfate from the glycosidic oxygen, presumed to be in contact with one of the active site residues, and the oxygen atom carrying the negative charge that is postulated to be in contact with Arg 424. Thus, in the absence of a crystallized enzyme-substrate complex, the approximate calculations indicate that the substrate molecule can be accommodated within the substrate cavity.

The second residue implicated in the α-β substrate specificity differences, α423Asparagine, does not appear to influence substrate specificity when introduced into a wild-type β subunit. The enhancement of 4MUGS hydrolysis by Asparagine at β452 in mutant Hex B carrying the Arginine substitution at position 453 is difficult to explain. Since the Asparagine residue is slightly larger than the Aspartate residue it is not likely that the positive effect of the Asp→Asn substitution is a result of decrease in steric hinderance. We considered an alternate hypothesis that in the β453Arginine mutant Hex B, the presence of a negatively charged Aspartate in the adjacent position on the peptide chain influenced the rotation around the peptide bond, thus changing the position of βArg453 relative to the three active-site amino acids. However, a comparison of the linear distances between

αArg424 and the three active site amino acids in models containing either Asp or Asn at position 423 shows no significant differences for the five measurements (see Figure 2).

The molecular determinants of the remainder of the substrate specific activity of the Hex A α-subunit remain to be defined. Nevertheless, we believe that our investigation indicates the manner in which residues important for substrate specificity will be discovered. The process will involve 1) inspection of the molecular models of the catalytic sites of the α and β subunits to identify amino acids whose side chains project into the substrate cavity, 2) identification of positions at which the two polypeptides have non-homologous amino acids, 3) selection of a list of candidates which may favor or prevent binding of electronegative substrates because of electrostatic, steric, or hydrophobic factors, 4) introduction of candidate α-subunit amino acids into the corresponding β subunit positions, and 5) evaluation of the substrate specificity of mutant forms of Hex B.

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