Direct Determination of the Substrate Specificity of the α-Active Site in Heterodimeric β-Hexosaminidase A†

Yongmin Hou,‡,§ Roderick Tse,‡,§ and Don J. Mahuran*,‡,§

Research Institute, Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada, and Department of Clinical Biochemistry, University of Toronto, Toronto, Ontario M5G 2C4, Canada

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ABSTRACT: The β-hexosaminidase isozymes are produced through the combination of α and β subunits to form any one of three active dimers (monomeric subunits are not functional). Heterodimeric hexosaminidase A (αβ) is the only isozyme that can hydrolyze G_{M2} ganglioside *in vivo*, requiring the presence of the G_{M2} activator protein. Hexosaminidase S (αα) exists but is not considered a physiological isozyme. Although hexosaminidase B (ββ) is present in normal human tissues, it has no known unique function *in vivo*. However, a unique function for the β-active site present in both hexosaminidase A and B has been indicated in a previous study of the various substrate specificities of the homodimeric forms of hexosaminidase (S and B). It was concluded that the α-active site is only able to efficiently hydrolyze negatively charged substrates, and the β-active site is only able to hydrolyze neutral substrates. When this model of nonoverlapping α- and β-substrates is extrapolated to heterodimeric hexosaminidase A, it has a major effect on the interpretation of recent results relating to the mode of action of the G_{M2} activator protein. In this report, we directly examine these substrate specificities using a novel form of hexosaminidase A containing an inactive β subunit, produced in permanently transfected CHO cells. We demonstrate that, whereas the β-active site has the same substrate specificities in either its A-heterodimeric or B-homodimeric forms, the α-active site in the A-heterodimer has different kinetic parameters than the α-active site in the S-homodimer. We conclude that the α and β subunits in hexosaminidase A participate equally in the hydrolysis of neutral substrates.

Two major β-hexosaminidase isozymes (Hex A and Hex B)[1] are detectable when assayed in normal human tissues using a common neutral artificial substrate, MUG. Hex A is a heterodimer composed of an α and a β subunit. The α subunit is encoded by the *HEXA* gene mapped to chromosome 15q23-q24 (Nakai et al., 1991). The β subunit is encoded by the *HEXB* gene mapped to chromosome 5q13 (Bikker et al., 1988). Hex B is a homodimer of β subunits. The near total homology in the placement of the intron/exon junctions and the close similarity in the deduced primary structures between the *HEXA* and *HEXB* genes and the α and β subunits, respectively, indicate that they have a common evolutionary origin (Korneluk et al., 1986; Proia, 1988). Thus, the subunits likely share many structure−function relationships. A third, unstable isozyme, Hex S, was found to be composed of two α subunits. All three Hex isozymes have nearly identical M_r, 122000−127000 (Mahuran & Lowden, 1980). Hex S was originally detected and characterized in human cells that do not synthesize a functional β subunit, *i.e.* from Sandhoff patients (see below) (Ikonne et al., 1975). More recently, it has been generated in other mammalian cells by transfection with the human α-cDNA, *e.g.* monkey kidney COS cells (Navon & Proia, 1989; Tanaka et al., 1990a,b). The expression of Hex S in these latter studies was used as a method for analyzing naturally occurring mutations in the HEXA gene causing Tay-Sachs disease. Two other inheritable diseases with clinically similar phenotypes are Sandhoff disease and the AB-variant form of G_{M2} gangliosidosis. In these cases, the disease arises from mutations in either the *HEXB* gene or the *GM2A* gene (encoding the G_{M2} activator protein), respectively. The predominant characteristic in all three of these disorders is the storage of G_{M2} ganglioside in lysosomes, primarily those of neuronal cells [reviewed in Gravel et al. (1995)]. Since above normal levels of Hex B are present in patients with Tay-Sachs disease and low but significant levels of Hex S are present in patients with Sandhoff disease, it is apparent that (a) similar but not identical active sites are contained in both subunits and (b) the only isozyme that can hydrolyze G_{M2} ganglioside *in vivo* is heterodimeric Hex A. In order for Hex A to perform this function, it requires the small, heat stable G_{M2} activator protein (activator). The exact role of the activator remains somewhat controversial. However, it is generally agreed that the activator interacts with both the carbohydrate and lipid portion of the ganglioside, solubilizing or at least lifting a ganglioside molecule from the membrane and "presenting" it to Hex A for hydrolysis (Meier et al., 1991). In 1984, an

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* To whom correspondence should be addressed at Research Institute, Hospital For Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada, Telephone: 416-813-6161. Fax: 416-813-5086.

‡ Hospital for Sick Children.

§ University of Toronto.

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[1] Abbreviations: MUG, 4-(methylumbelliferyl)-β-*N*-acetylglucosamine; MUGS, (methylumbelliferyl)-β-*N*-acetylglucosamine-6-sulfate; MU, methylumbelliferone; G_{M2} ganglioside, GalNAcβ(1−4)-[NeuAc(2−3)-]Galβ(1−4)-Glc-ceramide; NeuAc, *N*-acetylneuraminic acid; ER, endoplasmic reticulum; Hex, β-hexosaminidase; wt, wild type; β*, the β subunit of β-hexosaminidase carrying a Arg211Lys substitution mutation; activator, G_{M2} activator protein; FCS, fetal calf serum.

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α-specific artificial substrate, MUGS (Bayleran et al., 1984), was developed. The idea for such a substrate came from two earlier reports which demonstrated that Hex A and Hex S but not Hex B catalyzed the release of GlcNAc-6-sulfate from certain glycosaminoglycans (Bearpark & Stirling, 1978; Kresse et al., 1981).

All the above observations suggested that only Hex isozymes containing an α subunit as part of the dimer were capable of hydrolyzing negatively charged substrates. This model was later modified by Kytzia and Sandhoff (1985). In their report, they first confirmed kinetically that there were distinct active sites contained within the α and β subunits of Hex A. More importantly, they concluded that the active sites of the α (as measured in Hex S) and β (as measured in Hex B) subunits differed markedly in their K_m values toward neutral, MUG (Hex S, 2.8 mM; Hex B, 0.91 mM), as well as toward negatively charged, MUGS (Hex S, 0.33 mM; Hex B, 3.4 mM), artificial substrates. These data from the homodimers were then extrapolated to Hex A, and a model for the spectrum of substrates recognized by its α- and β-active sites was proposed. In this model, the α-active site is responsible for the hydrolysis of negatively charged substrates, and the β-active site is primarily responsible for the hydrolysis of neutral substrates.

Both the α and β subunits are synthesized in the ER as larger precursor polypeptides and are processed to their mature forms in the lysosome [reviewed in Gravel et al. (1995)]. Exit from the ER to the Golgi requires that the proteins acquire a near native conformation and that they form dimers (Proia et al., 1984). Many point mutations that cause Hex A deficiencies result in the retention of the mutant protein in the ER [reviewed in Mahuran (1991)]. An interesting exception to point mutations that exclusively affect folding is the αArg178His substitution associated with the B1 variant of Tay-Sachs disease (Kytzia et al., 1983; Ohno & Suzuki, 1988; Tanaka et al., 1988). The B1 variant has an unusual phenotype. Patient samples contain both the A and B isozymes; however, the Hex A lacks activity toward MUGS and G_{M2} ganglioside. In a previous report, we analyzed the biochemical consequences of the B1 substitution in the α subunit by *in vitro* mutagenesis of the homologous codon in the β-subunit, βArg211His. We found that the substitution did not affect dimer formation or cellular targeting but caused a near total loss of activity toward MUG. Consistent with the initial report of this substitution and its computer-predicted effects on secondary structure [reviewed in Suzuki and Vanier (1991)], we noted small additional changes in the stability and the rate of processing of the mutant protein (Brown et al., 1989). Although there was no re-activation of the mutant Hex B protein, these additional effects were totally eliminated when a more conservative substitution, βArg211Lys, was made. Through kinetic studies and the use of Arg-specific modifying agents, we were able to conclude that αArg178 and βArg211 are active site residues, *i.e.* part of the catalytic sites, in Hex (Brown & Mahuran, 1991).

The catalytic sites of other exoglycosidases, *e.g.* β-galactosidase and glucocerebrosidase (Gebler et al., 1992; Miao et al., 1994), are composed of two active acidic residues. However, a recent report suggests that an active basic and an active acidic residue comprise the catalytic site of Hex. This conclusion was based on the observation that the compound (±)-6-acetamido-1,2-anhydro-6-deoxymyoinositol (*N*-acetylconduramine B *trans*-epoxide) is a tight-binding inhibitor and pseudosubstrate for *N*-acetyl-β-glucosaminidases, *i.e.* Hex, from both bovine kidney and jack bean. With other exoglycosidases whose substrates are sugars that do not contain an *N*-acetyl group, this type of inhibitor forms a covalent bond with one of their active acidic residues (Legler & Bollhagen, 1992).

In the almost thirty years since the first description of the two major Hex isozymes (Robinson & Stirling, 1968), there have been no reports identifying an active Hex monomer. All reported data indicate that dimerization is necessary for either the α- or β-active sites to become functional [reviewed in Gravel et al. (1995) and Mahuran et al. (1985)]. Thus, dimer formation likely has a significant effect on the three-dimensional structures of the active sites contained within each subunit. Additionally, ganglioside hydrolysis studies have provided evidence that the identity of the subunit paired with the α subunit (αα or αβ) can have an effect on its ability to bind substrate. In *in vitro* assays, detergent, *e.g.* sodium taurocholate, can be substituted for the activator protein. Under these conditions, Hex S, as well as Hex A, but not Hex B can efficiently hydrolyze G_{M2} ganglioside. Interestingly, Hex B can hydrolyze G_{A2}, the neutral, asialo derivative of G_{M2}, in the presence of detergent but not in the presence of activator. Also, the G_{M2}/activator complex acts as an inhibitor toward Hex S hydrolysis of MUGS. These data suggest that both the binding site for the complex and the catalytic site for G_{M2} hydrolysis are located in the α subunit. However, binding of the β subunit to the α subunit is necessary for production of the complex-binding site that can correctly orientate the β-GalNAc group of G_{M2} into the α-catalytic site [reviewed in Furst and Sandhoff (1992) and Sandhoff et al. (1989)].

The above observations suggest that the extrapolations of kinetic data from Hex S to the α subunit active site of Hex A may not be completely valid. However, since no method had been devised that would make possible the study of the α-active site of Hex A in isolation, these extrapolations have been generally accepted. Here, we report a new molecular method for production of a Hex A with our previously characterized, inactive β subunit (β*Arg211Lys; Brown & Mahuran, 1991). We use the modified isozyme, partially purified from permanently cotransfected CHO cells, to directly analyze the kinetic properties of the α subunit in its most important *in vivo* form, heterodimeric Hex A.

MATERIALS AND METHODS

DNA Construction. In order to create the pREP4-α construct, a 2.0-kilobase (kb) *Bam*HI fragment containing α-chain cDNA was isolated from pSVL-α (Brown & Mahuran, 1993) and ligated into the *Bam*HI site of the mammalian expression vector pREP4 (InVitrogen), which contains the hygromycin B gene as a selective marker. To obtain the wild type pEFNEO-β, the 2.0 kb *Bam*HI partially digested fragment containing the entire β-chain cDNA from pHexB43 (Brown et al., 1989) was initially ligated into pSL301 (InVitrogen), designated pSL301-β. The 2.0 kb *Eco*RI/*Not*I fragment containing the β-cDNA from pSL301-β was then subcloned into the expression vector pEFNEO (kindly supplied to us by Dr. Anson) (Anson et al., 1992), which has a neomycin (G418) resistance marker. To generate the mutant pEFNEO-βArg211Lys, a 2.0 kb frag-

ment, partially digested by *Bam*HI from pCDβArg211Lys (Brown & Mahuran, 1991), was isolated and subcloned into the *Bam*HI site of the pEFNEO-β vector. The mutation was verified by DNA sequencing.

Cell Culture and DNA Transfection. CHO cells were grown in α-MEM with 10% FCS and antibiotics at 37 °C in 5% CO_2. Transfections were performed according to the Lipofection reference manual from GIBCO-BRL. Briefly, CHO cells were seeded onto 100 mm tissue culture dishes and grown overnight until they were about 60% confluent. Then 20 μg of pREP4α and 10 μg of wt pEFNEO-β (wt Hex A) or mutant pEFNEO-βArg211Lys (αβ*-Hex A) were mixed with 70 μg of Lipofectin reagent (GIBCO BRL) in 200 μL of serum-free MEM. The mixture was allowed to sit for 30 min at room temperature to form the DNA−Lipofectin complex. One milliliter of serum-free medium was added to the mixture prior to its being added dropwise to the culture dishes. After a 5 h incubation period at 37 °C, 5 mL of α-MEM plus 10% FCS was added to the dishes. The next day, the cells were washed and refed with α-MEM plus 10% FCS for 2 days. Following this, the cells were trypsinized and replated at a 1/10 dilution in α-MEM plus FCS containing 400 μg/mL each of neomycin and hygromycin A. After 2 weeks of growth, drug-resistant colonies were picked and put in 24-well plates with media. The medium and lysate from the surviving cells were assayed for Hex activity, and those producing the highest levels were selected for further growth and analyses. Both neomycin and hygromycin (each 400 μg/mL) were present in all the stable transfectants.

Hex Activity Assay. Cells were lysed in a buffer of 10 mM Tris-HCl (pH 7.5) and 5% glycerol through five sets of freeze−thaw cycles. Protein from cell lysate was quantitated by the Lowry method. Hex activity from cell lysates and media was determined using a α-chain-specific substrate MUGS and the common substrate MUG (Brown & Mahuran, 1993).

Western Blot Analysis. Equal amounts of total proteins from each sample (or equal volumes from DEAE fractions, see below) of transfected and nontransfected CHO cell lysates were resolved by polyacrylamide gel electrophoresis in sodium dodecyl sulfate by the Laemmli gel system (12.5% gel) using a Bio-Rad mini-gel system (Laemmli, 1970). Proteins were transferred to nitrocellulose overnight at 4 °C. The filter was blocked in 5% skim milk and then incubated overnight with a 1/800 dilution (1% skim milk) of rabbit anti-human Hex A. Nitrocellulose was washed four times with 1% skim milk and was incubated with a 1/10000 dilution (1% skim milk) of horseradish peroxidase/conjugated goat anti-rabbit IgG for 1 h. The filter was developed and exposed to Hyperfilm using the Amersham ECL system.

Separation of Hex Isozymes by DEAE Ion-Exchange Chromatography. Proteins (5 mg) from control CHO or transfected cell lysates were applied to a 3 mL column of DEAE CL-6B (Pharmacia). The unbound Hex B fraction was collected by washing the column with 10 mM sodium phosphate (pH 6.0). To remove most of the endogenous CHO Hex A, the column was washed with 50 mL of 0.075 M NaCl in 10 mM sodium phosphate (pH 6.0). In order to separate Hex A from Hex S, a 0.075 to 0.25 M NaCl gradient was used. Fractions (3 mL) were collected and assayed for Hex activity. The concentration of salt in each fraction was determined by conductivity measurements. The Hex A (p*I* 4.8) peak eluted at ∼0.1 M NaCl, whereas the more acidic Hex S (p*I* 3.6) peak eluted at ∼0.2 M NaCl.

Kinetic Analysis. The K_m and V_{max} values for αβ-Hex A, αβ*-Hex A, and Hex S were determined by varying the concentration of the MUG substrate from 0.4 to 4 mM for either form of Hex A and from 0.4 to 7.5 mM for Hex S. When MUGS was used as a substrate, its concentration was varied between 0.2 and 1.6 mM. The αβ-Hex A, αβ*-Hex A, and αα-Hex S that were used were purified from transfected CHO cells by DEAE ion-exchange chromatography (see above). Hex S from cell lysates of fibroblasts from a patient with infantile Sandhoff disease, *i.e.* line GM294 shown to be homozygous for a 5′ deletion of the *HEXB* gene (Neote et al., 1990; O'Dowd et al., 1986), was also used as a further control. Kinetic constants were calculated using a computerized nonlinear least squares curve-fitting program for the Macintosh, KaleidaGraph 3.0. Thus, the individual substrate concentrations and their corresponding initial velocity measurements were directly fitted to the Michaelis−Menten equation, $V_i = V_{max}[S]/(K_m + [S])$, making possible the calculation of an accurate standard error (Tommasini et al., 1985a). The V_{max} values were calculated from the theoretical maximum nanomoles of MU per hour reached in each kinetic experiment by division by the amount of Hex−protein used. This value was calculated on the basis of the specific activity (MUG hydrolyzed per milligram) of each isozyme purified from human placenta, *i.e.* Hex A = 6.4 and Hex S = 1.0 mmol h^{-1} mg^{-1}, using a corrected E_{280} of 1.11 OD_{280} mg^{-1} cm^{-1} (Mahuran & Lowden, 1980).

G_{M2} Hydrolysis. G_{M2} ganglioside (20 nmol), tritium-labeled in its *N*-acetylgalactosamine moiety, was incubated in 10 mM citrate buffer (pH 4.1) with the same number of MUGS units produced by human cDNA-derived Hex A, Hex A*, or Hex S or endogenous CHO cell Hex A, 0.5% human serum albumin, and 10 mM GlcNAc (carrier) in a final volume of 100 μL in the presence of 2.0 μg of activator protein purified from transformed bacteria (Klima et al., 1993) or 10 mM detergent sodium taurocholate (Novak & Lowden, 1980). The reaction mixture minus the Hex sample was utilized as the negative control. The reaction was performed at 37 °C over 18 h and stopped with 1 mL of chloroform and 1 mL of 12 mM citrate/phosphate buffer (pH 4.1) containing 13.6 mM GlcNAc. The hydrolyzed product from G_{M2}, *i.e.* [^{3}H]GalNAc, was separated from the unreacted G_{M2} substrate by passage through a positively charged ion-exchange minicolumn of 0.6 mL of AG3 X4 (acetate form) resin. The unbound fraction containing [^{3}H]GalNAc was determined by liquid scintillation counting.

RESULTS

Two CHO cell lines were established, each from a clonal population of cells selected for their similar high level of Hex (MUGS) expression (data not shown). One line was permanently transfected with the wild type (wt) α-cDNA and wt β-cDNA, while the other was transfected with the wt α-cDNA and the βArg211Lys-encoding mutant β-cDNA (β*).

Western blots of the cotransfected cell lysates demonstrated the presence of mature (lysosomal) forms of both subunits (α and either β or β*, data not shown). Total MUG activity was 24-fold higher in the α and β-cDNA-transfected

Table 1: Separation of the Hex Isozyme Activities[a] Contained in Lysates[b] from Nontransfected CHO Cells (None) and CHO Cells Cotransfected with cDNAs Encoding wt α and wt β or wt α and Mutant β−Arg211Lys (β*) by DEAE Ion-Exchange Chromatography[c]

CHO cells + DNA	substrate used	total sample Hex A+ B + S		0.0 M NaCl Hex[d] B		0.075 M NaCl Hex[d] A + B		0.1 to 0.12 M NaCl Hex[d] A		% recovery
		units[a]	*G/S*[e]	units	*G/S*	units	*G/S*	units	*G/S*	
none	MUG	6.4	−	2.1	−	2.3	−	1.1	−	86
none	MUGS	1.6	4.0	0.03	70	0.8	2.9	0.3	3.7	71
α and β	MUG	156	−	57	−	12	−	56	−	80
α and β	MUGS	19	8.2	0.2	290	1.9	6.1	14	4.0	85
α and β*	MUG	38	−	2.5	−	3.3	−	25	−	83
α and β*	MUGS	20	1.9	0.06	42	1.6	2.1	13	1.9	73

[a] Units of μmol of MU h^{-1} mL^{-1}. [b] Equal amounts of lysate protein (5 mg) from transfected and nontransfected cells were used for the individual separation. [c] See Figure 1. [d] The Hex isozymes (see Figure 2) contained in the given pool of ion-exchange column fractions eluting in the above range of NaCl concentrations (see Figure 1). [e] The ratio of units of MUG to those of MUGS hydrolyzed.

cells than in nontransfected cells (Table 1, α and β, and none, total). The α- and β-cDNA-transfected cells were also 4-fold higher in MUG activity than were the α- and β*-cDNA-transfected cells (Table 1, α and β, and α and β*, total). Similarly, MUGS activity was 12-fold higher in α- and β-cDNA-transfected cells than in the negative control cells but had a level nearly identical to that of the α- and β*-cDNA-transfected cells (Table 1, total). In one respect, the comparisons of activities produced by the α- and β-cDNA- and α- and β*-cDNA-transfected cells are not surprising since the CHO cell clones were selected on the basis of their similar high levels of MUGS activity. On the other hand, the observation that MUG activity was only decreased by 4-fold in the α- and β*-cDNA-transfected cells was unexpected, suggesting that the α-active site in αβ*-Hex A must have significant activity toward the neutral MUG substrate.

To further confirm the above hypothesis, the Hex isozymes were separated by DEAE-Sepharose ion-exchange chromatography, a technique well established for this purpose (Mahuran et al., 1985; Mahuran & Lowden, 1980). The isozymes in each of three cell types were analyzed by at least three independent separations to ensure reproducibility, with representative elution profiles shown in Figure 1 for (a) negative control cells, (b) cloned CHO cells expressing α and β, and (c) cloned CHO cells expressing α and β*. The data from these separations are summarized in Table 1. Under the conditions used, human Hex B is not bound by the column and elutes in the 0.0 M NaCl wash, and human Hex A (0.10 to 0.12 M) and Hex S (0.19 to 0.21 M) are separated by elution with a NaCl gradient (Mahuran & Lowden, 1980) (Figure 1). To confirm the presence of the mature subunits (indicative of lysosomal incorporation) in the various fractions, Western blotting was performed. As expected in cells transfected with either α- and β- or α- and β*-cDNAs, only mature wt β or β* subunits were detected in the 0 M NaCl wash (despite the lack of a significant increase in MUG activity in this fraction from cells transfected with the α- and β*-cDNA as compared to that of the nontransfected cells, Table 1), and both mature α and β or β* subunits were present in the 0.1 to 0.12 M NaCl pools (Figure 2). A small more acidic peak was also detected at about 0.19 to 0.21 M NaCl (Figure 1) that contained only α subunits (data not shown) corresponding to Hex S. It was found that about 75% of the endogenous CHO cell Hex A, *i.e.* MUGS activity, could be removed by a 0.075 M NaCl wash (Table 1, none, 0.075 M) prior to beginning the NaCl gradient (compared to 12% of the human-derived MUGS activity; Table 1, α and β, 0.075 M), effectively raising the levels of both MUG and MUGS activities in the human Hex

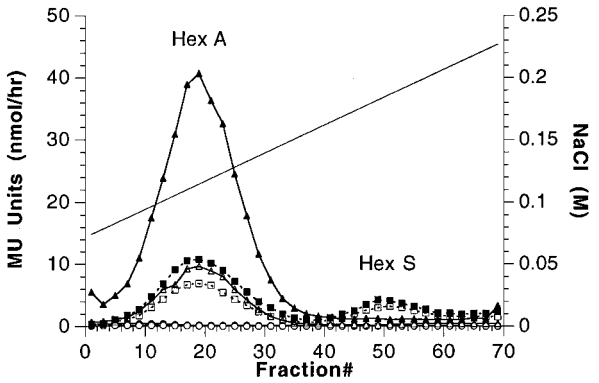

FIGURE 1: DEAE ion-exchange separation (0.075−0.25 M NaCl) of the Hex isozymes from CHO cells cotransfected with the following: (a) wild type α- and β-cDNA (solid lines), closed triangles are MU produced from the common MUG substrate and open triangles are MU produced from the α-specific MUGS substrate; and (b) cDNA encoding wild type α and Arg211Lys substitutes β* (dotted lines), closed squares are MU produced from the common MUG substrate and open squares are MU produced from the α-specific MUGS substrate. Hex B was eluted in the 0 M NaCl wash and >75% of the endogenous CHO cell Hex A wash eluted in a 0.075 M NaCl wash (Table 1). Residual CHO Hex activities eluting in the NaCl linear gradient are shown in closed (MUG) and open (MUGS) circles and were undetectable in unconcentrated fractions.

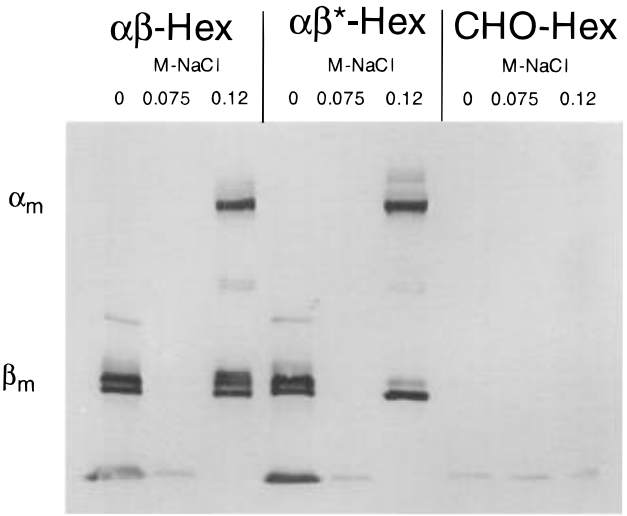

FIGURE 2: Western blot analysis of the concentrated pools of fractions from the DEAE column (Figure 1). The identities of the subunits producing the immunoreactive bands with the anti-Hex A antiserum are indicated on the left and are of the expected M_r (mature α = 56000, mature β = 26−30000).

A fractions (Table 1, αβ, 0.1 to 0.12 M) to 50-fold above negative control levels (Table 1, none, 0.1 to 0.12 M). The levels of residual activity eluting within the 0.1 to 0.12 M

Table 2: K_m and V_{max} Values for wt (αβ) and Mutant (αβ*) Hex A Compared with Those for Hex S (αα) Utilizing MUG and MUGS Substrates

Hex	MUG K_m[a]	MUG V_{max}[a]	MUGS K_m[a]	MUGS V_{max}[a]	MUG K_m[b]	MUG V_{max}[b]	MUGS K_m[b]	MUGS V_{max}[b]
αβ	0.73 ± 0.05	10.3	0.24 ± 0.05	2.00	0.90	10.8	0.31	1.50
αβ*	0.92 ± 0.05	5.39[c]	0.21 ± 0.05	2.06	ND[f]	ND	ND	ND
αα[d]	1.53 ± 0.04	1.62	0.29 ± 0.07	0.91	2.8	1.02	0.33	0.34
αα[e]	1.50 ± 0.08	1.98	0.29 ± 0.03	0.94	ND	ND	ND	ND

[a] Data from this report. The K_m values for each substrate are given in millimolar with the standard error reported as ±, calculated by the KaleidaGraph 3.0 model fitting Macro (directly fitting the initial velocity versus substrate concentration data to the Michaelis−Menten equation). The V_{max} values are given as mmol of MU h^{-1} mg^{-1}; the amount of Hex−protein analyzed was calculated on the basis of the specific activity (Mahuran & Lowden, 1980). [b] Data from Kytzia and Sandhoff (using human liver Hex A and B) (Kytzia & Sandhoff, 1985); V_{max} values were recalculated to mmol h^{-1} mg^{-1}. [c] The specific activity was calculated from the isozyme's MUG/MUGS hydrolysis ratio of 1.9 as compared to 4.0 for αβ-Hex A (Table 1). [d] Hex S derived from human fibroblasts from a patient with infantile Sandhoff disease. [e] Hex S produced in transfected CHO cells and separated by DEAE chromatography (Figure 1). [f] Not Determined.

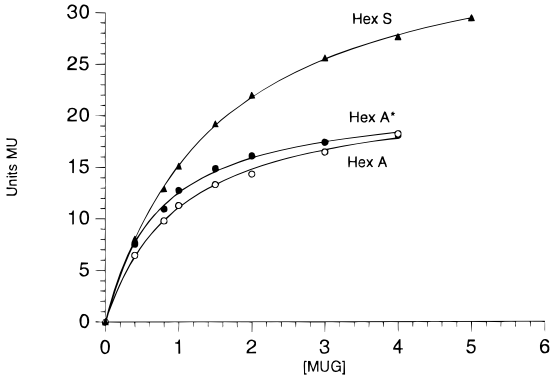

FIGURE 3: Kinetic analysis of the Hex isozymes using the neutral MUG (millimolar) substrate. The experimental data points are shown and were directly fitted to the Michaelis−Menten equation. The equation with its calculated best-fit constants (K_m and apparent V_{max}) were then used to generate the lines joining the data points on the graph. The R values for all of the experiments were >0.985. The actual K_m and V_{max} values are given in Table 2.

NaCl gradient from negative control cell lysates were so low as to be undetectable in individual, unconcentrated DEAE fractions (Figure 1). Thus, the ion-exchange separation results in the isolation of the expressed human Hex A that is >98% free of contamination by the CHO isozyme. This separation was also improved upon by our use of only the fractions from 0.11 to 0.12 M NaCl. We deemed that our preparations of human wt αβ-Hex A and αβ*-Hex A were sufficiently free from the endogenous CHO isozyme to allow us to undertake substrate specificity and kinetic studies.

The following is clear from the data given in Table 1. (a) The human Hex B has little activity toward MUGS, producing a MUG/MUGS (*G*/*S*) ratio of ∼300/1.0 (α and β, 0 M NaCl). (b) A comparison of the *G*/*S* ratio of αβ- and αβ*-Hex A eluted between 0.1 and 0.12 M NaCl indicates that the α-active site is responsible for the hydrolysis of about 50% of the neutral MUG substrate. It is also apparent that the α-active site of αβ*-Hex A can hydrolyze MUG at about twice the rate of the negative MUGS substrate when the same concentration of each substrate is present (1.6 mM). (c) Whereas the ratio of MUG/MUGS hydrolysis was ∼4/1 for αβ-Hex A and ∼2/1 for αβ*-Hex A (Table 1, 0.1 to 0.12 M NaCl, *G*/*S*), it was only ∼1/1 for Hex S (Figure 1). These data suggest some differences in the α-active site when it is present in the heterodimeric versus the homodimeric form.

To further examine the role of the α-active site in the hydrolysis of neutral substrates, the apparent K_m values were determined for the MUG and MUGS substrates using αβ-Hex A, αβ*-Hex A, and αα-Hex S [from a Sandhoff fibroblast cell line as was previously done (Kytzia & Sandhoff, 1985) and from transfected CHO cells (Figure 1)]. Table 2 summarizes the kinetic data. The apparent K_m values (Figure 3) utilizing wt Hex A and Hex S are slightly different but generally consistent with the measurements made by Kytzia et al. (Kytzia & Sandhoff, 1985). From these data alone, it would be predicted that the α subunit does not play a large role in the hydrolysis of the neutral substrates *in vivo*. However, when the heterodimeric αβ*-Hex A was examined, the K_m for the MUG substrate was found to be similar to that of wt Hex A (Figure 3) and Hex B (Brown & Mahuran, 1991) from either transfected CHO cells, human placenta (Tommasini et al., 1985b), or human liver (Kytzia & Sandhoff, 1985), *i.e.* 0.7−0.9 mM. Thus, the composition of the Hex dimer has a significant effect on the substrate specificity of the α subunit active site.

Table 3: G_{M2} Ganglioside Hydrolysis

Hex	(nmol of G_{M2}/h)/U_{MUGS} + 2 μg of activator[a]	(nmol of G_{M2}/h)/U_{MUGS}+ 10 mM sodium taurocholate[b]
αβ	(1.3 ± 0.1) × 10^{-3} [e]	(5.3 ± 0.2) × 10^{-4}
αβ*	(1.2 ± 0.3) × 10^{-3}	(4.8 ± 0.2) × 10^{-4}
αα[c]	(0.000 ± 0.001) × 10^{-3}	ND[f]
CHO(−)[d]	(0.000 ± 0.001) × 10^{-3}	ND[f]

[a] 25, 50, and 100 MUGS units (nmol of MU h^{-1}) were used for 18 h incubations. [b] 100, 200, and 400 MUGS units (nmol of MU h^{-1}) were used for 1 h incubations. [c] Hex S derived from human fibroblast lysates from a patient with infantile Sandhoff disease. [d] MUGS units derived from nontransfected CHO cell lysates. [e] Values are the slope of the best-fit line generated by plotting the units of MUGS used versus units of ganglioside hydrolyzed (nmol of G_{M2} h^{-1}) ± the standard error of the slope. [f] Not Determined.

The effect of an inactive β* subunit on the ability of Hex A to hydrolyze its natural substrate, G_{M2} ganglioside, in the presence of the human G_{M2} activator protein was analyzed. Equal numbers of MUGS units from αβ-Hex A, αβ*-Hex A, Hex S, or nontrasfected CHO cell lysate (endogenous Hex A) were used. In the presence of equal amounts of human activator protein, nearly identical amounts of G_{M2} ganglioside were hydrolyzed by either αβ-Hex A or αβ*-Hex A (Table 3). However, under identical conditions and similar MUGS units of human Hex S or endogenous CHO Hex A, we failed to detect any G_{M2} hydrolysis (Table 3). These data provide further evidence that the native conformation of the Hex A isozyme has not been affected by the βArg211Lys substitution in αβ*-Hex A. Because there is no significant increase in G_{M2} hydrolysis with the wt Hex A, the data also confirm earlier evidence from the study of the B1 variant of Tay-

Sachs disease that the β-active site in Hex A cannot interact in a productive manner with the terminal β-linked GalNAc residue in the G_{M2} ganglioside/G_{M2} activator complex.

The β-active site, when present in its homodimeric Hex B form, has also been shown to be inactive toward G_{M2} ganglioside in the presence of the activator and only slightly active in the presence of detergent (Hex S can hydrolyze G_{M2} ganglioside in the presence of detergent but not in the presence of activator alone) [reviewed in Sandhoff et al. (1989)]. To determine if the β-active site in Hex A behaves in the same manner, equal MUGS units of $\alpha\beta$*-Hex A and wt Hex A were incubated with G_{M2} ganglioside in the presence of sodium taurocholate. As with the above experiment with activator protein, nearly identical levels of G_{M2} ganglioside were hydrolyzed (Table 3). These data indicate that the presence of a functional β-active site does not significantly enhance the ability of Hex A to hydrolyze G_{M2} ganglioside even in the presence of a nonspecific detergent.

DISCUSSION

The similar clinical phenotypes produced from *HEXA* defects (encoding the α subunit of Hex A), Tay-Sachs disease, and *HEXB* defects (encoding the β subunit of Hex A), Sandhoff disease, indicate that the heterodimeric Hex A ($\alpha\beta$) isozyme is the most important *in vivo* form of Hex. Since each subunit has its own active site, requiring dimerization to become functional, the direct study of either of the active sites in Hex A has not been possible. Instead, the characteristics of each active site in the heterodimer have been assumed to be identical to their characteristics in Hex homodimers, *i.e.* Hex B ($\beta\beta$) and Hex S ($\alpha\alpha$). Whereas Hex B is present in normal human tissues along with Hex A, Hex S is only present in easily detectable (but still very small) amounts in samples from patients with Sandhoff disease (O'Dowd et al., 1986). Thus, Hex S is not a normal physiological Hex isozyme, and it is likely that attribution of properties to the α-active site in Hex A on the basis of extrapolations from the study of Hex S may be misleading. In this study, we directly determine some of the kinetic parameters of the α-active site contained in a form of Hex A constructed with an inactive βArg211Lys subunit. The identification of βArg211 as an active residue and the properties of the mutant β subunit were documented in two previous reports (Brown & Mahuran, 1991; Brown et al., 1989).

The data we present in this report are fully consistent with previous conclusions relating to the β-active site in Hex A as extrapolated from studies of Hex B, *i.e.* it has little activity toward either MUGS (Table 1) or G_{M2} ganglioside (Table 3). We show that binding of the mutant β subunit to the wt α subunit has no effect on the ability of Hex A* to hydrolyze MUGS (as compared to wt Hex A, Table 3). Given these data, the actual weight of $\alpha\beta$*-Hex A can be calculated to produce V_{max} values (Table 2) from the known specific activity (MUG) of purified wt Hex A (Mahuran & Lowden, 1980), the *G*/*S* ratio of the $\alpha\beta$*-Hex A isozyme (Table 1), and the MUGS units used in the kinetics experiments. V_{max} values for Hex S can also be calculated from its specific activity which was determined after purified placental Hex A was converted to Hex S by merthiolate treatment and isolated by DEAE ion-exchange chromatography (Mahuran & Lowden, 1980). Our V_{max} data for MUG were 3.3-fold higher for the heterodimeric $\alpha\beta$*-Hex A form which contains only one active α subunit than for the homodimeric Hex S form containing two active α subunits. Thus, the α-active site in $\alpha\beta$*-Hex A is 7-fold more catalytically active toward neutral MUG than either of the α-active sites in Hex S.

The clarification of the substrate specificity of the α subunit of Hex A is fundamental for the interpretation of the specificity of the G_{M2} activator protein. Data have recently been presented by Wu et al. that the activator's role goes beyond the simple detergent-like effect on G_{M2} ganglioside. They concluded that the activator also acts to reduce the hydrogen bonding between the acetamido NH of the terminal GalNAc residue and the carboxyl group of the NeuAc (linked to the internal Gal residue), freeing the GalNAc residue for hydrolysis (Wu et al., 1994). This conclusion was based on the difference in the ability of Hex A to hydrolyze negatively charged G_{M2} ganglioside and its neutral asialo derivative (lacking the NeuAc group), G_{A2}. The argument was made that, if a specific function of the activator is to promote the hydrolysis of the branching saccharide structure present in G_{M2} (but not G_{A2}), then the addition of activator would have a smaller effect on Hex A's ability to hydrolyze G_{A2}. The report went on to demonstrate experimentally that there was little enhancement of Hex A's ability to hydrolyze G_{A2} as compared to its ability to hydrolyze similar amounts of G_{M2}, by the addition of 0.5 μg of activator to the assay mixture (Wu et al., 1994). However, older reports by Sandhoff and colleagues indicate a large increase in Hex A activity toward G_{A2} with the addition of activator protein (Conzelmann & Sandhoff, 1978, 1979). The data we present in this report have important implications for future experiments aimed at resolving this conflict. If the α subunit in Hex A had the substrate specificity, previously reported by Kytzia and Sandhoff on the basis of the analysis of Hex S (Table 2) (Kytzia & Sandhoff, 1985), one would not expect significant G_{A2} hydrolysis in the presence of the activator because of the inability of this neutral substrate, complexed with the activator, to interact efficiently with the α-active site. However, our findings of a similar K_m and $\sim 0.5 V_{max}$ for the hydrolysis of neutral MUG (Table 2, Figure 3) by $\alpha\beta$*-Hex A (one active subunit) as compared to that of $\alpha\beta$-Hex A (two active subunits) demonstrate that the α- and β-active sites in Hex A are equally as efficient at hydrolyzing neutral substrates. Whereas these are necessary data to validate the experimental design of Wu et al., they do not in themselves resolve the above conflict.

Our data also call into question the use of Hex S expression as a method for analysis of naturally occurring mutations in the HEXA gene causing Tay-Sachs disease, particularly if the mutation is suspected of affecting the active site. The novel method presented in this report utilizing CHO cell coexpression of the wt α with an inactive β subunit may prove to be a valuable method for analysis of such mutations, *i.e.* those associated with a B1-variant phenotype. In these cases, the lack of the potentially confounding activity produced by an active β subunit would clearly be of help in differentiation between a mutation that affects substrate specificity and one that directly affects catalytic activity.

Finally, the data suggest that there is no unique *in vivo* requirement for a functional β-active site. Like $\alpha\beta$*-Hex A, the α-active site of wild type Hex A should be able to hydrolyze all the Hex isozymes' natural substrates. The

requirements for the β subunit are restricted to components of its protein structure. These unidentified structural components act to (a) stabilize the Hex A heterodimer, allowing efficient exit of the enzyme from the ER and transport to the lysosome; (b) activate the α-active site in the dimer, making it functional toward both neutral and negatively charged substrates; and (c) promote the binding of the G_{M2} ganglioside/activator protein complex in the correct orientation. None of these roles can be fulfilled through the formation of the α-homodimer, Hex S.

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