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Osiecki-Newman, Karen Marie

HUMAN ACID BETA-GLUCOSIDASE: FUNCTIONAL AND STRUCTURAL STUDIES OF THE NORMAL AND GAUCHER DISEASE ENZYMES

City University of New York PH.D. 1987

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Human Acid B-Glucosidase: Functional and Structural Studies of the Normal and Gaucher Disease Enzymes

by

Karen M. Osiecki-Newman

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1987

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

Human Acid B-Glucosidase: Functional and Structural Studies of the Normal and Gaucher Disease Enzymes.

by

Karen M. Osiecki-Newman

Advisor: Dr. Gregory A. Grabowski

Comparative enzymatic studies of the human acid β-glucosidase (β-Glc; N-acyl-sphingosyl-1-O-β-glucoside:glucohydrolase, E.C.3.2.1.45) from normal placentae and spleens of Type 1 Ashkenazi Jewish Gaucher disease (AJGD) patients were conducted. Novel affinity chromatographic techniques were developed to obtain homogeneous normal acid β-Glc and highly purified AJGD enzyme. Affinity labeling of the pure normal β-Glc with the covalent, active site-directed inhibitor, conduritol B epoxide (CBE), demonstrated the presence of a single active site per subunit and permitted the identification of the active site nucleophile necessary for catalysis, Asp^{443}. Studies of active site function determined that the AJGD enzyme had: 1) a single active site per subunit; 2) nearly normal k_{cat} and k_{max} values for several substrates and CBE, respectively, as well as normal relative V_{max} values for glucosyl ceramides with increasing (C_1 to C_{18}) fatty acid acyl chain lengths; 3) five to seven-fold increased $K_i$app values for particular C1 and C5 amino substituted glucosides and alkyl-glucosides (C_0 to C_{14}) derivatives, but normal $K_i$app values for the most potent glycon inhibitors, nojirimycin and castano-

spermine; and 4) normal $K_m$app values for all substrates. These results were consistent with the active site of the normal enzyme being composed of three domains: a glycon binding domain which recognizes the glycon moieties of substrates and inhibitors and contains Asp^{443} and a critical amino acid with pK_a=6.7; an aglycon binding domain which interacts with acyl moieties and modulates V_{max}; and a "third" domain which binds alkyl moieties and modulates the affinity of substrates and inhibitors. These studies also indicate that the mutation in the AJGD variant results in a unique localized effect on β-Glc active site function.

Foreword

Portions of this thesis have been presented in the following publications:

Osiecki-Newman, K.M., Fabbro, D., Dinur, T., Boas, S., Gatt, S., Legler, G., Desnick, R.J. and Grabowski, G.A.: Human Acid β-Glucosidase: Affinity Purification of the Normal Placental and Gaucher Disease Splenic Enzymes on N-Alkyl-Deoxynojirimycin-Sepharose. Enzyme 35:147-153, 1986. Permission granted by S. Karger AG, Basel, Switzerland.

Dinur, T., Osiecki, K.M., Legler, G., Gatt, S., Desnick, R.J. and Grabowski, G.A.: Human Acid β-Glucosidase: Isolation and Amino Acid Sequence of a Peptide Containing the Catalytic Site. Proc. Natl. Acad. Sci. USA 83:1660-1664, 1986.

Grabowski, G.A., Osiecki, K.M., Dinur, T., Kruse, J., Legler, G. and Gatt, S.: Gaucher Disease Types 1, 2 and 3: Differential Mutations of the Acid β-Glucosidase Active Site Identified with Conduritol B Epoxide Derivatives and Sphingosine. Am. J. Hum. Genet. 37:499-510, 1985.

Grabowski, G.A., Osiecki-Newman, K.M., Dinur, T., Fabbro, D., Legler, G., Gatt, S. and Desnick, R.J.: Human Acid β-Glucosidase: Use of Conduritol B Epoxide Derivatives to Investigate the Catalytically Active Normal and Gaucher Disease Enzymes. J. Biol. Chem. 261:8263-8269, 1986.

Osiecki-Newman, K., Legler, G., Grace, M., Dinur, T., Gatt, S., Desnick, R.J. and Grabowski, G.A.: Human Acid β-Glucosidase: Inhibition Studies Using Glucose Analoques and pH Variation to Characterize the Normal and Gaucher Disease Glycon Binding Domains. Arch. Biochem. Biophys., in review.

Osiecki-Newman, K., Fabbro, D., Legler, G., Desnick, R.J. and Grabowski, G.A.: Human Acid β-Glucosidase: Use of Inhibitors, Alternate Substrates and Amphiphiles to Investigate the Properties of the Normal and Gaucher Disease Active Sites. Biochim. Biophys. Acta, in review.

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I wish to express my heartfelt thanks to my family for their patient support and understanding during the course of these studies. And finally, my sincerest appreciation goes to my husband, Jeffrey, for his encouragement and love and for making this "the best of all possible worlds".

Table of Contents

*Please note: This thesis is divided into distinct chapters; each is a separate publication or body of work. The references for each are collated separately and are compiled by chapter in the section, Literature Cited.

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List of Abbrevtations

Gaucher disease	GD
Acid β-glucosidase	β-Glc
Ashkenazi Jewish Gaucher disease	AJGD
High performance liquid chromatography	HPLC
Cross-reacting immunologic material	CRIM
Acetonitrile	ACN
Trifluoroacetic acid	TFA
Sodium dodecylsulfate	SDS
Polyacrylamide gel electrophoresis	PAGE
Critical micellar concentration	CMC
Human or bovine serum albumin	HSA or BSA
Absorbance at 280 nm or 214 nm	A^{280}; Abs^{280} or A^{214}; Abs^{214}
Concentration of inhibitor which results in 50% inhibition of the activity	I_{50} or IC_{50}
Glucosyl ceramide (N-acyl-sphingosyl-1-O-β-glucosid⁻)	GC
Glucosyl ceramide with fatty acyl chain length of n carbons	C_n-GC
Glucosyl sphingosine	GS
Conduritol B epoxide (1-D-1,2-anhydro-myo-inositol)	CBE
Bromo-conduritol B epoxide (6-bromo-6-deoxy-1-D-1,2-anhydro-myo-inositol)	Br-CBE
Bromo-tritiated-conduritol B epoxide	[^{3}H]Br-CBE or [^{3}H]Bromo-CBE
Deoxynojirimycin (1,5-dideoxy-1,5-imino-D-glucitol)	dNM

N-alkyl-deoxynojirimycin with alkyl chain length of n carbons	(N-)C_n-dNM
4-methylumbelliferyl-1-O-β-D-glucopyranoside	4MU-Glc
4-alkyl-umbelliferyl-1-O-β-D-glucopyranoside the alkyl chain varies from n=1 to n=11	4-C_n-U-Glc
[N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazo-4-yl)]	NBD
NBD-dodecanoyl-GC	NBD-C_{12}-GC
NBD-hexanoyl-GC	NBD-C_6-GC
(3-trifluoromethyl)-3-(m-[^{125}I]iodophenyl) diazirine	[^{125}I]-TID

Introduction

Gaucher disease is an autosomal recessive disorder which is characterized by the deficiency of acid β-glucosidase (β-Glc; D-glucosyl-N-acylsphingosine glucohydrolase, E.C.3.2.1.45) (1,2,3). The lysosomal accumulation of glucosyl ceramide (GC), the primary substrate of β-Glc (4,5), within cells of the monocyte/macrophage system results in the bone marrow expansion, boney deterioration, hypersplenism and hepatic involvement characteristic of this disease (1). Gaucher disease (GD) has been classified into three major subtypes based on the absence or presence (and severity) of neuronal involvement (6). Type 1 GD is a non-neuronopathic disease with a high prevalence in the Ashkenazi Jewish population (gene frequency = 0.02) (7), although this form has been reported in several ethnic groups (8,9). Type 1 GD is the most prevalent Jewish ethnic disease based on the known gene frequency. The onset of clinical manifestations in this subtype is highly variable. Type 2 GD is a rare, panethnic disease with onset in infancy and an acute neuronopathic course. Type 3 GD is a subacute neuronopathic form with juvenile onset which was first described in Norrbotten, Sweden (10). The high incidence of consanguinity in the Type 3 GD (11,12) and the lack of complementation of Type 1 and Type 2 GD fibroblasts (13) provide support for the existence of different allelic mutations in this disease. Although the basis for the clinical variability observed between and within the subtypes and variants has been the subject of speculation (1), the nature of the β-Glc defects which would account for these differences remains unknown. The focus of this thesis was to elucidate the nature of the basic enzymologic defect in Type 1

AJGD by comparing the properties of the mutant enzyme to those of the normal β-Glc.

Purification and Characterization of Acid β-Glucosidase

Requisite to understanding the alterations in the mutant enzymes in the GD subtypes is knowledge of the structure and function of normal β-Glc. Such studies required the development of methods to purify the enzyme. Pentchev et al. (14) employed conventional methods to obtain a ~4000-fold purification of the normal splenic enzyme in a 5% yield. The use of hydrophobic chromatography permitted large scale preparation of a highly purified placental β-Glc with a ~30% overall yield (15). Concanavalin A-Sepharose (16), phosphatidylserine-Agarose (16) and natural β-Glc protein effector affinity supports (17) were used to obtain highly purified preparations of β-Glc. These methods were hindered by support ligand contamination of the final preparation (15), nonspecificity of binding and the unavailability of the ligand (18), respectively. Several investigators (18,19) employed glucosphingosine (GS), a potent inhibitor of β-Glc, as an affinity ligand. Use of these affinity supports did not provide homogeneous enzyme and the resultant enzyme preparations were unstable. Grabowski and Dagan (18) synthesized a GC support which yielded apparently homogeneous β-Glc. However, the degradation of the support and loss of binding capacity after multiple uses were major drawbacks and required the difficult resynthesis of the support.

Purification methods using HPLC molecular weight separation techniques (20,21) were unable to resolve β-Glc from major contaminants

under the conditions employed and the methods were not quantitative. More recently, Choy (22) developed a two-step high performance procedure using hydrophobic and gel permeation column chromatography for a 10,000-fold purification and a 37% overall yield. This protocol represented the first preparative-scale HPLC method available, although the specific activity of the final preparation was 3-fold less than several of the previously described affinity methods. The development of efficient and reproducible methods for normal β-Glc purification as well as for purifying the defective β-Glc activity from GD tissues for comparative studies were a priority of the present investigations.

Previous investigations (14,15) have shown that the purified normal placental enzyme is a homomer with a subunit molecular weight of 67,000 Da. Several studies (23,24,25) have demonstrated that β-Glc from a variety of tissues has multiple forms with pI values ranging between 3.2 to 6.5. The purified placental enzyme was resolved into several forms with pI values of 4.2 to 6.2 by isoelectric focusing (26). Sequential treatment of the purified placental enzyme with glycosidases resulted in one major form (pI = 7.8) (26) indicating the multiple forms are due to glycosylation. The structures of the oligosaccharides on the placental β-Glc are typical biantennary and triantennary mannosyl cores which contain sialic acid, and N-acetylglucosamine (27). The exact number of oligosaccharide chains and their location on the polypeptide remain unknown. The predicted amino acid sequence of β-Glc from the cDNA indicated five Asn-X-Ser(Thr) consensus sequences for N-linked glycosylation (28). In addition, the mature, deglycosylated protein was deduced to be 497 amino acid residues corresponding to a molecular weight of 55,384 Da (29).

Few studies have detailed the kinetic properties of the normal human β-Glc. The enzyme has been shown to require detergents, negatively-charged lipids or a natural protein effector for optimal activity (30,31,32,33). Hyun et al. (34) employed a variety of inhibitors to demonstrate the presence and specificity of an "allosteric" site. Previous investigations which provided a basis for the present studies (35) have delineated three domains of the β-Glc active site: 1) the catalytic site which binds of glycon moieties of substrates and inhibitors; 2) the aglycon binding site which interacts with acyl moieties of particular inhibitors and substrates; and 3) the "third" domain which interacts with sphingosyl moieties as well as negatively-charged lipids. The term "domain" refers to a functional region or binding site in this instance and is not used in the classical sense which regards a domain as a contiguous sequence forming a three dimensional structure. Additional binding sites, not in the active site, may be required for interaction with the β-Glc protein effector (36). Vaccaro et al. (37,38) demonstrated reduced affinity of the enzyme for synthetic or hydrogenated GCs indicating a specificity for the sphingosyl double bond. Other studies (39) demonstrated the hydrolysis of GS by β-Glc and suggested overlapping, but not identical, binding sites for GS and GC. The present work focused on the elucidation of the domain function and structure as a basis for understanding the defective function of β-Glc in the Gaucher disease subtypes and variants.

Characterization of the Gaucher Disease Acid β-Glucosidases

The occurrence of clinically distinct phenotypes of Gaucher

disease and the detection of residual β-Glc activity in each subtype (1,12,40,41,42) suggests the presence of different mutant forms of β-Glc which retain partial activity. Several investigators reported that the levels of residual activity correlated with the clinical subtype (1,43) (i.e.: less residual activity results in more rapid accumulation of the substrate (GC) and a more severe clinical course of the disease). Other investigators have not confirmed this finding (44,45).

The residual enzyme in the GD subtypes and variants have been shown to exhibit altered physical, kinetic, and/or immunologic properties (40). Using the method of Turner et al. (46), previous studies by Grabowski et al. (40) demonstrated that the β-Glc from Type 1 AJGD fibroblasts was more heat labile than the normal enzyme. Karazeh et al. confirmed this finding (41). In addition, the residual activity from several non-Jewish Type 1 GD patients had normal, increased or decreased thermostabilities (40) evidencing the heterogeneity of the mutant gene products. Interestingly, the mutant gene products in all of the Gaucher disease subtypes and variants had normal K_m values for natural and artificial substrates and decreased V_{max} values (40). The enzymatic basis of these observations was a major focus of this thesis.

Qualitative differences between the residual β-Glc in the subtypes of GD have been demonstrated by the degree and magnitude of enzymatic responses to lipoidal modifiers (47,48,49). Using phosphatidylserine or gangliosides, Mueller et al. (50) showed that the residual enzyme in Type 1 GD fibroblasts had a 2-fold greater activation than either the normal or the Type 2 or 3 enzymes. Wenger et al. (51) and Glew et al. (52) achieved similar results using phosphatidylserine and the natural protein effector of β-Glc. Investigations by

Grabowski et al. (40), which were a basis for the present studies, examined the kinetic properties of the residual β-Glc activity in fibroblasts from a diverse group of GD patients. Three classes were delineated based on the response to a number of inhibitors and activators: 1) Group A residual activities were not distinguished functionally from the normal enzyme; 2) Group B, which consisted primarily of the Type 1 AJGD patients, exhibited elevated fold activation and elevated I_{50} values. These studies suggest an altered active site function of this residual β-Glc; and 3) the Group C patient was a possible genetic compound and the residual enzyme displayed intermediate responses. The elucidation of the altered interaction by the Type 1 AJGD residual activity was an aim of the present studies.

Immunological investigations have focused on the quantitation of the amount of CRIM and the correlation of "western blotting" profiles with the phenotype. Pentchev et al. (53), using polyclonal antibody raised to highly purified splenic β-Glc, found nearly normal amounts of CRIM in Type 1 fibroblasts and suggested a 16 to 20-fold decrease in the catalytic rate constant for the β-Glc from Type 1 GD spleen. More recent studies (54,55) have indicated CRIM levels of 30 - 100% of normal in Type 1 AJGD and that the CRIM specific activity (Unit activity/mg CRIM) was 8 to 10-fold decreased in this variant of GD. However, the interpretation of these studies was limited by the potential antigenic differences between the normal and mutant enzymes and the inability to determine the amount of catalytically active mutant enzyme in the preparations. Consequently, investigators used the "western blotting" technique to examine potential qualitative differences in molecular forms of β-Glc within and among the subtypes and

variants of GD (56,57). These studies indicated that the normal β-Glc and the Type 1 AJGD residual enzyme had three molecular forms (Mr = 67,000, 63,000, and 58,000 Da), but most of the non-Jewish Type 1 and Types 2 and 3 variants had single forms (Mr = 63,000 or 65,000 Da). Deglycosylation experiments (57) indicated that all the normal or mutant β-Glc forms detected with antibodies were derived from a polypeptide with Mr = 56,000 (i.e., the molecular weight of the mature polypeptide predicted from the cDNA (29)). The above physical, kinetic and immunological studies indicated that the mutant enzyme in the Type 1 AJGD variant resulted from an exonic mutation in the β-Glc structural gene.

Graves et al. (58), used Northern blot analysis and S1 nuclease mapping studies to show normal quality and quantity of mRNA from fibroblasts of GD patients. These results suggest that the mutations in the GD subtypes and variants are single base alterations. More recently, preliminary studies have identified a substitution in a Type 2 GD β-Glc genomic sequence which results in the creation of a restriction enzyme cleavage site (59). This altered site was found with a high frequency in the genomic DNA of patients with the neuronopathic forms of the disease (Types 2 and 3). However, the causal nature of the mutation and its relationship to the phenotype remains to be elucidated. In addition, the causal mutation in the Type 1 AJGD, the most prevalent form of the disease, still remains to be determined.

In the present studies, initial investigations were aimed to determining the basic nature of the normal β-Glc function and its structure. Particular effort was focused at ascertaining domains on the normal β-Glc with specific function (e.g., the catalytic site and

the membrane binding domain). The overall objective of these studies was the elucidation of the properties of the normal active site domains in an effort to understand the functional consequences of the mutation in Type 1 AJGD.

Chapter One

The Use of HPLC Techniques to Purify the Normal Placental Acid β-Glucosidase

Materials and Methods

The following were obtained from commercial sources: decyl-agarose, anti-human albumin and sodium cholate (Sigma, St.Louis, MO); octyl-sepharose (Pharmacia, Piscataway, NJ); 4-methylumbelliferyl-β-D-glucopyranoside (4MU-Glc; RPI, Mount Prospect, Ill); TSK SW 3000 column (7.5 x 305mm; Beckman Instruments, Inc., Somerset, NJ); ethylene glycol (Fischer Scientific, Fairlawn, NJ). The glucosphingosine-Sepharose was prepared as described (1). All other reagents were of the highest grade available.

The normal placental acid β-glucosidase (β-Glc), partially purified by hydrophobic chromatography (2) or by affinity chromatography on glucosphingosine-Sepharose (1), was isocratically eluted from a TSK SW 3000 column at 0.5 ml/min in 0.05M sodium phosphate, pH 6.0, containing 0.1N NaCl.

This preparation was diluted 20-fold with 0.05M sodium phosphate, pH 6.0, and concentrated by Amicon using a YM10 filter. The enzyme was re-applied and eluted isocratically from the TSK SW 3000 column in 0.05M sodium phosphate, pH 6.0, containing 10% ethylene glycol. Analytical SDS-polyacrylamide gel electrophoresis (12.5%) (3) and silver staining techniques (4) were used to assess the purity of the eluted enzyme. Immunoelectrophoretic techniques which were used to detect the presence of human serum albumin (HSA) were performed as described (5). Enzyme activities were determined fluorometrically with 4mM 4MU-Glc (6) and protein concentration were estimated by the Lowry procedure (7).

Results and Discussion

A typical purification of β-Glc on the TSK SW 3000 column is shown in Table 1. By exploiting the non-ideal behavior of the TSK SW 3000 column, this scheme resulted in normal placental β-Glc with a specific activity of 1.6×10^6 nmol/h/mg of protein in an overall yield of 7.2%. Although this column is generally used for size exclusion chromatography, the conditions of the first HPLC elution have been optimized to enhance β-Glc interaction with the solid phase. The solvophobic effects of this column have been well characterized (8) and increased ionic strength has been observed to enhance hydrophobic interactions between proteins and the solid phase. This property made possible the removal of the major contaminating protein of the enzyme preparations, HSA (1), which has the same molecular weight as β-Glc (Mr=67,000).

The enzyme, delayed in elution by interaction with the column, was typically contaminated by lower molecular weight proteins. These were subsequently separated by rechromatographing the preparation in buffer containing 10% ethylene glycol, as described above. The presence of ethylene glycol interferes with the non-ideal behavior of the column separating β-Glc and the contaminants. An elution profile is shown in Figure 1. The arrow indicates the peak with enzyme activity.

This procedure produced some of the highest specific activities observed to this date for β-Glc. The previous affinity method using glucosylceramide-Sepharose (1) produced nearly the same specific activities but, the difficulty in resynthesizing the support and the

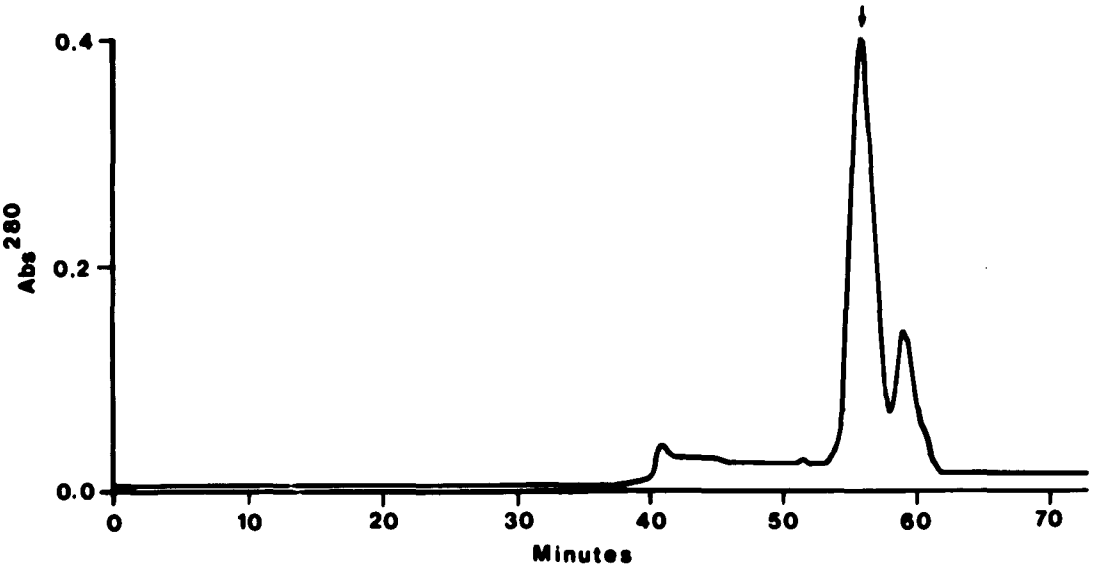
TABLE 1

Purification of Normal Acid β-Glucosidase

Purification Step	Specific Activity*	Yield
	(U/mg protein)	(%)
Homogenate/Cholate Extract	140	100
Ammonium Sulfate Supernatant	220	74.9
Acid Precipitate/Butanol Extract	2200	67.9
Decyl Agarose Eluate	180,000	65.9
Octyl Sepharose Eluate	390,000	47.1
HPLC (in NaCl)	563,000	10.6
HPLC (10% ethlyene glycol)	1,645,000	7.2

* U/mg protein = 1nmol of 4MU-Glc (4mM) hydrolyzed per h per mg of protein.

Figure 1



The elution profile of normal placental β-Glc from the TSK SW 3000 column in the presence of 10% ethylene glycol as monitored by Abs^{280}. The arrow indicates the peak with enzyme activity.

loss of binding capacity over time were major drawbacks. The commercial availability of the HPLC column provided an accessible alternative to the available affinity methods, although the column capacity was limited making this procedure time and labor intensive. Therefore, we continued to pursue efforts to obtain a more efficient scheme while employing this reproducible method for preparing pure and highly active β-Glc.

Chapter Two

Affinity Purification of the Normal Placental and Gaucher Disease Splenic Acid β-Glucosidases on N-Alkyl-Deoxynojirimycin-Sepharose

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Materials and Methods

Materials:

The following were from commercial sources: decyl agarose and sodium cholate (Sigma, St. Louis, MO); octyl-Sepharose and aminohexyl-Sepharose 4B-CL (Pharmacia, Piscataway, NJ); Protein C-4 column (4.6 x 250 mm/l; The Nest Group, Southboro, MA); 4-methylumbelliferyl-β-D-glucopyranoside (4MU-Glc; RPI, Mount Prospect, IL). Acetonitrile and trifluoroacetic acid were HPLC grade. All other reagents were of the highest grade available.

The N-alkyl-dNM supports were synthesized from N-(10-carboxydecyl)dNM (C_{10}-dNM) or N-(12-carboxydodecyl)dNM (C_{12}-dNM) by carbodiimide dehydration with aminohexyl-Sepharose (17). The ligand concentrations were 4 or 7 μmol/ml of settled gel for C_{10}-dNM or C_{12}-dNM, respectively (17).

Methods:

Enzyme Preparation:

Normal β-Glc from human placentae was partially purified by hydrophobic chromatography (13) as modified by Grabowski and Dagan (4). The Type 1 Ashkenazi Jewish Gaucher disease (AJGD) β-Glc was purified from spleen which had been obtained at splenectomy and was frozen at -20°C until use. The residual enzyme in fibroblasts and splenic extracts from this patient had the physical and kinetic properties characteristic of this form of Gaucher disease (18,19). For enzyme purification from the Type 1 AJGD spleen, the procedure of Grabowski and Dagan (4) was modified as follows: 1) the citric acid precipitation

was eliminated to avoid major losses (> 85%) of the mutant enzymatic activity and 2) the supernatant from the ammonium sulfate step was delipidated with 20% butanol. The dialyzed butanol extract of the Type 1 AJGD spleen or the normal enzyme obtained from the ethylene glycol elutant of the octyl-Sepharose column (4) was used for subsequent affinity purification procedures.

Analytical Procedures:

Enzyme activities were determined fluorometrically with 4 mmol/l 4MU-Glc (7) and protein concentrations were estimated by the Lowry procedure (20). Reverse phase HPLC and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were as described (8). Capacities of the affinity columns were estimated (4). Amino acid composition and sequence analyses were accomplished by Dr. Kenneth Williams of the Protein Chemistry Facility in the Department of Biophysics and Biochemistry at Yale University.

Results and Discussion

Affinity Purification of Normal β-Glc:

Table 2 summarizes typical purifications of β-Glc from 10 kg of human placentae by C_{10}- and C_{12}-dNM chromatography. The enzyme was purified about 20,000-fold from the crude homogenate (9500-fold from the cholate extract) with an overall yield of 14 to 18%. The final enzyme preparations from either column had similar specific activities (1.4 to 1.7 x 10^6 nmol/h/mg protein using 4 mmol/l 4MU-Glc as substrate) and were stable in 60% ethylene glycol at 4°C for four to six

TABLE 2

Purification of Acid β-Glucosidase from Normal and Type 1 AJGD Tissues

Purification Step	Normal		Type 1 AJGD	
	Specific Activity*	Yield	Specific Activity*	Yield
	(U/mg protein)	(%)	(U/mg protein)	(%)
Cholate extract	170	100	4.5	100
35% Ammonium sulfate	380	70	9.9	63
Acid ppt/Butanol extract	160	43	4.0[a]	51
Decyl agarose	137,000	45	—	—
Purification 1:				
Octyl Sepharose I	338,000	40	—	—
N-C_{10}-dNM Sepharose	1.6×10^6 [b]	14	26,500	10.9
Purification 2:				
Octyl Sepharose II	306,000	45		
N-C_{12}-dNM Sepharose A	948,000	30		
N-C_{12}-dNM Sepharose B	1.7×10^6	18		

* U = nmol of 4MU-Glc (4 mmol/l) hydrolyzed per h.

[a] Acid precipitation (ppt) was omitted.

[b] Final specific activity ranged from 1.46 to 1.74×10^6 U/mg protein.

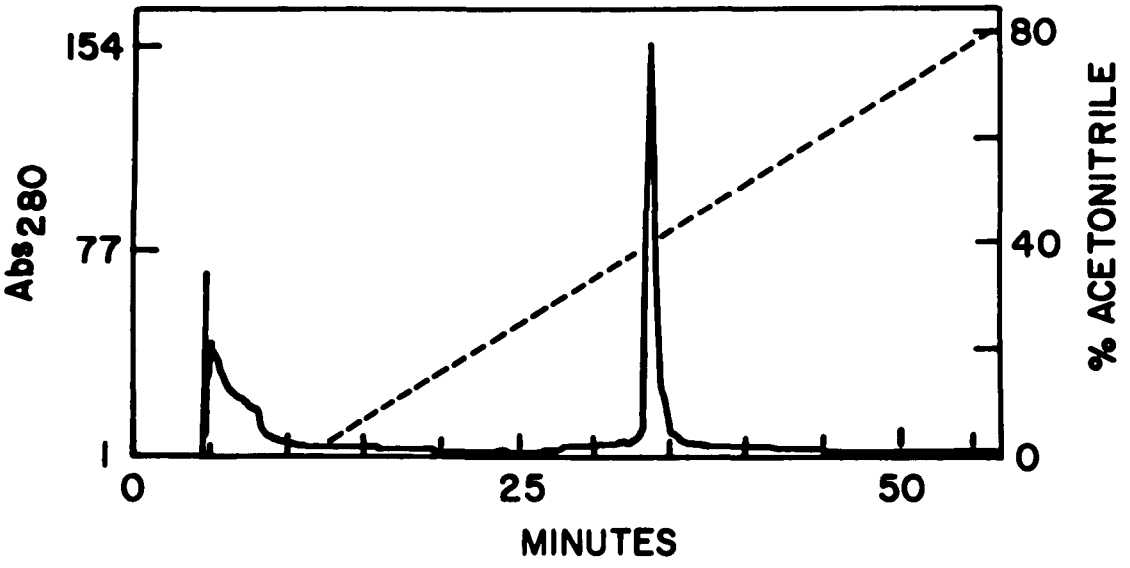
months. The homogeneity of these preparations was evidenced by a single protein species on SDS-PAGE gels detected by the silver-staining technique (data not shown), and detected on reverse phase HPLC (Fig. 2). In addition, these enzyme preparations had a single N-terminal amino acid sequence. The first 22 residues are shown below with tentative assignments in parentheses and unidentifiable residues designated by X:

Ala-Arg-Pro-X-Ile-Pro-Lys-Ser-Phe-Gly-Tyr-
Ser-Ser-Val-Val-X-(Val)-X-X-Ala-Thr-Tyr....

This sequence was colinear with that predicted from the human β-Glc cDNA nucleotide sequence (3) and indicates the junction of the leader piece and the mature N-terminus. Amino acid composition analyses of β-Glc (Table 3) revealed 35% hydrophobic amino acids (Ile + Leu + Met + Val + Aromatics) which was in good agreement with the composition predicted from the cDNA (3).

The placental β-Glc, partially purified by hydrophobic chromatography, was diluted with 0.05 mol/l citrate, pH 5.5, containing 4 mmol/l β-mercaptoethanol and 5 mmol/l EDTA (buffer A) to an ethylene glycol concentration of < 5% and applied (1 ml/min) to equilibrated columns of either C_{10}- or C_{12}-dNM-Sepharose. The columns were then subjected to sequential washes (100 column vol each) with buffer A, buffer A containing 1% taurocholate and then, buffer A alone. The enzyme then was eluted from either affinity support at about 40 to 60% ethylene glycol in a 10 to 90% linear gradient in buffer A (Fig. 3). The enzyme eluted from the C_{12}-dNM support typically contained variable amounts (5 to 15%) of a contaminant ($M_r \cong 47,000$) which could be re-

Figure 2



Reverse phase HPLC profile of normal placental β-Glc purified by C_{10}-dNM-Sepharose. Five nmoles (280 µg) of β-Glc were applied, and then eluted at 41% acetonitrile in 0.05% trifluoroacetic acid. The peaks at 5 to 10 min were due to absorbing materials in the buffer.

TABLE 3

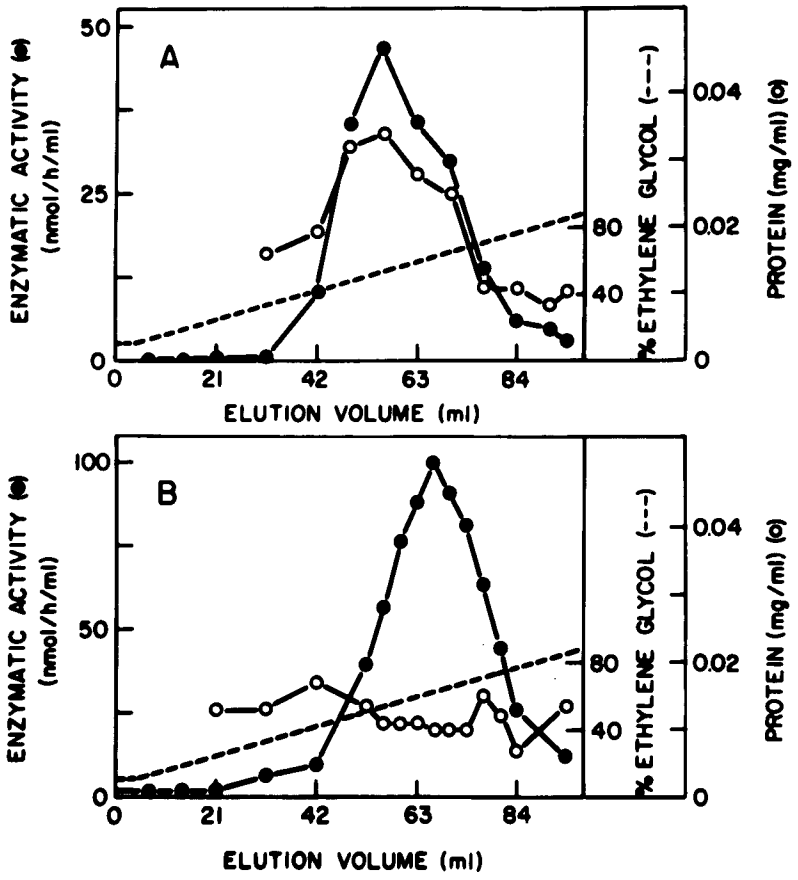
Amino Acid composition of β-Glc

Amino Acid	Number of Residues*
Asx**	48
Thr	28
Ser	29
Glx**	43
Pro	34
Gly	39
Ala	43
Val	32
Met	8
Ile	21
Leu	58
Tyr	19
Phe	28
His	18
Lys	24
Arg	26
Cys	—
Trp	—

* Based on MW = 55,382

** Asx = Asp + Asn; Glx = Glu + Gln

Figure 3



Elution profile of normal (A) and Type 1 AJGD (B) β-Glc activity from C_{10}-dNM-Sepharose. Enzyme activity (●) and protein (O) eluted coincidentally in a 10 to 90% ethylene glycol gradient (---) after extensive washes (see text). The enzyme activities in A were multiplied by 10^{-3}.

solved from β-Glc by reapplication followed by elution with a 40 to 90% ethylene glycol gradient in buffer A (Table 2). In comparison, the enzyme obtained from C_{10}-dNM-Sepharose was homogeneous (Fig. 2). The capacities of the affinity supports were about 0.5 to 0.75 mg β-Glc/ml of settled gel and remained unchanged without regeneration (4) through ten uses of either column.

Importantly, all solutions and buffers as well as the affinity columns must contain 4 mmol/l β-mercaptoethanol to maintain enzyme stability. At β-mercaptoethanol concentrations between 0.5 and 3.0 mmol/l, the enzyme activity had a $t_{1/2}$ of 0.1 to 0.5 h, whereas at 4 mmol/l β-mercaptoethanol, the β-Glc activity was stable for 4 to 6 months. Interestingly, when the enzyme activity of a homogeneous preparation was allowed to decrease (in 2 mmol/l β-mercaptoethanol) to a specific activity of 750,000 nmol/h/mg protein and, then, β-mercaptoethanol was added to 4 mmol/l, the activity no longer decreased with time. The original specific activity (1.5×10^6 nmol/h/mg protein) of homogeneous β-Glc could be fully restored by repurifying the above preparation on C_{10}-dNM. About 50% of the homogeneous protein which was inactive was obtained from the 1% taurocholate wash of the affinity column. In contrast, about 50% of the homogeneous protein was eluted with the ethylene glycol gradient. This latter protein had a specific activity typical of pure β-Glc. These results suggest that only active enzyme bound to the affinity support and was eluted specifically with ethylene glycol. Thus, this procedure provides a method to obtain purified preparations which contain only active enzyme. This observation was supported by finding a 1:1 mol/mol stoichiometry of β-Glc protein and [^{3}H]Br-CBE (8). These findings also may account for the higher

specific activities of these preparations than those obtained with a substrate affinity ligand method (4,15).

Affinity Purification of Type 1 AJGD β-Glc:

Table 2 also summarizes the purification of β-Glc from 4 kg of Type 1 AJGD spleen by C_{10}-dNM chromatography. The final preparation was about 7500-fold enriched over that in the crude homogenate (about 5900-fold over the cholate extract). This preparation was obtained in an 11% yield and several protein bands were detected on silver stained SDS-PAGE gels (data not shown). Only a band at $M_r \cong 67,000$, which corresponded to the pure normal glycosylated placental β-Glc, was detected by immunoelectroblotting using polyclonal or monoclonal antibodies to normal β-Glc (21). Reapplication and elution of the final preparation to the C_{10}-dNM column resulted in recoveries of 10 to 15% of the applied enzymatic activity and insufficient protein was obtained for analysis. In buffer A containing 60% ethylene glycol and 4 mmol/l β-mercaptoethanol, the enzyme activity was stable for at least five months.

To obtain yields greater than 1% of the original Type 1 AJGD enzymatic activity, the acid precipitation and hydrophobic chromatography steps (4,10,13) were eliminated. The cold (4°C) butanol extract, obtained after dialysis against 2% butanol (4) and containing 4 mmol/l β-mercaptoethanol, was applied directly to a 5 ml column of C_{10}-dNM (1 ml/min). The washes and elution were exactly the same as for normal placental β-Glc except that the mutant enzyme activity in the crude splenic extract eluted at 50 to 70% ethylene glycol (Fig. 2). Extensive comparative kinetic studies of the normal and Type 1 AJGD β-Glc pre-

parations indicated that the mutant β-Glc had a specific active site defect(16).

In summary, two new affinity columns employing potent [K_i = 50 to 100 nmol/l (16)] β-Glc inhibitors have been shown to be useful for purification of normal and Type 1 AJGD β-Glc. Since pilot studies (unpublished observation, G.A. Grabowski) have indicated that the N-alkyl-dNM supports bind other glucosidases (e.g., neutral β-glucosidase and acid α-glucosidase isozyme 1 and 2), the affinity columns might be adapted to the sequential purification of these enzymes by differential elution with specific substrates or inhibitors. For β-Glc, these affinity supports provide for a high yield of homogeneous normal enzyme which retains full catalytic activity and should facilitate investigations of this enzyme's structure and function. In addition, the C_{10}-dNM support provided a simple, rapid and higher yield alternative to the low yield, multi-procedural method (22) for purifying mutant β-Glc from Gaucher disease spleen.

Chapter Three

Isolation and Amino Acid Sequence of a Peptide Containing the Catalytic Site of Acid β-Glucosidase

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Materials and Methods

Materials:

The following were obtained commercially: human and bovine serum albumin (Sigma Chemical Co., St. Louis, MO); 4-methylumbelliferyl β-D-glucopyranoside (4MU-Glc; Research Products International, Mount Prospect, IL); NBD-dodecanoic acid (Molecular Probes, Junction City, OR); Staphylococcus aureus V_8 Protease (V_8 protease, Miles Scientific, Napierville, IL); Vydac™ Protein C-4 column (4.6 x 250 mm; The Nest Group, Southboro, MA); Protosol™ (New England Nuclear, Boston, MA). Acetonitrile and trifluoroacetic acid were HPLC grade. All other chemicals were reagent grade or better.

GC was purified from spleens of Gaucher disease patients (21) and NBD-dodecanoyl-GC was prepared from glucosylsphingosine (22). Br-CBE, [^{3}H]Br-CBE (8000 cpm/pmol) and N-alkyl-deoxynojirimycin-Sepharose were synthesized as described (23,24).

Enzyme Purification, [^{3}H]Br-CBE Labeling and Proteolytic Cleavage:

Human acid β-glucosidase was purified to homogeneity by affinity chromatography using N-alkyl-deoxynojirimycin-Sepharose (24). Aliquots (1-2 nmol) of the purified enzyme were labeled with [^{3}H]Br-CBE as follows: the enzyme, in 0.5 ml of buffer A (0.04 M citrate/0.05 M phosphate, pH 5.5, containing 4 mM β-mercaptoethanol and 5 mM EDTA) and 60-80% ethylene glycol, was incubated at 22°C for 96 hr with a 10-fold molar excess (0.15 ml) of [^{3}H]Br-CBE (800 cpm/pmol). Control experiments indicated that the enzyme was stable under these conditions. To

this solution, sufficient 1.25 M Tris-HCl, pH 6.8, 5% sodium dodecyl sulfate (SDS) and concentrated ethylene glycol were added to achieve the following final concentrations: 0.125 M Tris/HCl, pH 6.8, 0.5% SDS, and 50% ethylene glycol (buffer B). This solution then was heated to 100°C for 2 min. After cooling to 22°C, freshly prepared V_8 protease in buffer B (2-8 µl) was added to a 17-fold excess of enzyme protein (wt/wt) and incubated at 37°C for 96 hr. Control experiments demonstrated that [^{3}H]Br-CBE did not bind to V_8 protease. V_8 protease in the digests was inactivated by heating at 100°C (2 min) in the presence of 2% β-mercaptoethanol for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or by freezing at -20°C prior to reverse phase HPLC.

Isolation of Radiolabeled Peptides:

The V_8 protease digests of the radiolabeled acid β-glucosidase were defrosted and subjected to reverse phase HPLC (Waters Associates) using a Protein C-4 column in 0.05% trifluoracetic acid. The peptides were eluted with a programmed nonlinear 0-80% acetonitrile gradient (see Results) at a flow rate of 0.7 ml/min. In pilot runs the gradient was optimized to provide maximal resolution of the peptides. The eluted absorbance peaks (A^{280} or A^{214}) were collected in teflon tubes and the radioactivity determined in aliquots from each tube. Analytical SDS-PAGE (12.5%) (25) and the silver staining technique (26) were used to monitor the purity of the peptides. The amino acid sequence of one of the pure radiolabeled peptides was determined using gas-phase sequencing techniques (27). Hydropathy profiles and probable secondary structural assignments were calculated using the data of Kyte and Doolittle (28) and the program of Corrigan and Huang (29),

respectively.

Quantitation of Acid β-Glucosidase Active Sites:

The relationship between the amount of enzymatic protein (pmol) and [^{3}H]Br-CBE bound to the enzyme was determined by incubating various amounts of active homogeneous acid β-glucosidase with a large excess of [^{3}H]Br-CBE (10 μM final concentration) in buffer A containing 0.6% human serum albumin. For these studies the enzyme was diluted with buffer A to an ethylene glycol concentration of <1%. Control studies demonstrated that under these conditions human serum albumin was required to maintain enzyme stability at 22°C for up to 24 hr. Complete inactivation of the enzyme by [^{3}H]Br-CBE was achieved by 2 hr at 22°C. To ensure that all sites which could bind [^{3}H]Br-CBE were saturated, the reaction mixture was allowed to stand at 22°C for 24 hr. The enzyme-[^{3}H]Br-CBE complexes were immunoprecipitated quantitatively with monospecific rabbit anti-human acid β-glucosidase IgG and Staphylococcus aureus Protein A (12). The resultant supernatants were reprecipitated with additional IgG and Protein A until no additional increase in precipitated radioactivity was observed; immunoprecipitation usually was quantitative after a single cycle. The resultant pellets were washed four times by resuspension and centrifugation (10,000xg; 40 min) in phosphate buffered saline containing 1% bovine serum albumin, 0.5 M NaCl and 0.05% Tween 20 (x2) and then in phosphate buffered saline containing 0.05% Tween 20 (x2). The washed pellets were dissolved in 100 μl of Protosol™ in 900 μl of water (24 hr at 22°C) and the radioactivity determined. The data are presented as the pmol of homogeneous enzymatic protein based on the protein concentration (30) and the

estimated molecular weight (55,000) for the unglycosylated enzyme subunit as calculated from the amino acid composition (20,24). These results were in close (± 10%) agreement with calculations of the enzymatic protein concentration (pmol) based on the turnover number (140 nmol substrate hydrolyzed/hr/pmol enzyme) of the homogeneous enzyme using 4MU-Glc as substrate (31).

Determination of the I_{50} values for Br-CBE:

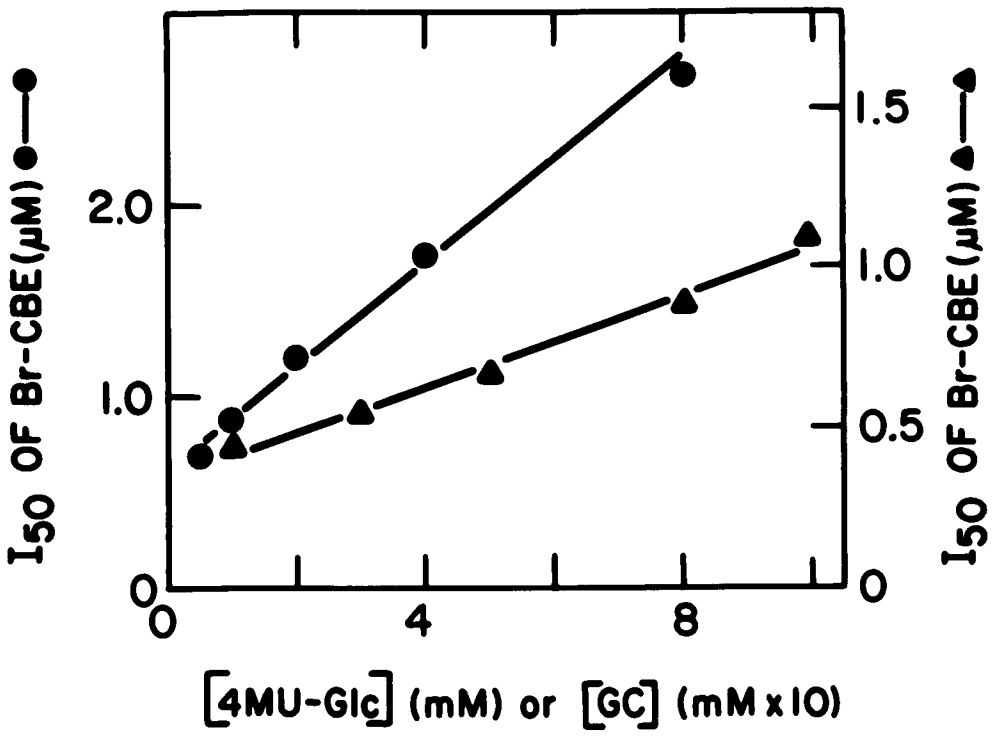
The I_{50} values for Br-CBE, i.e., the concentration of Br-CBE required to achieve 50% inhibition of the original enzymatic activity, were determined as follows: the final incubation mixture (0.2 ml) contained 0.04 M citrate/0.05 M phosphate, pH 5.5, 4 mM β-mercaptoethanol, 5 mM EDTA, 4 mM Triton X-100, 4.65 mM sodium taurocholate and the required amounts of Br-CBE (11) and 4MU-Glc or GC [NBD-dodecanoyl-GC: splenic GC; 1:19, mol/mol (22)]. Reactions were initiated by the addition of homogeneous enzyme in amounts determined to hydrolyze less than 2% of the substrate. After 1 hr the reactions were terminated and the fluorescence intensity of the products determined (22).

Results

Interaction of Br-CBE and Acid β-Glucosidase:

Figure 4 shows the direct dependence of the I_{50} values for Br-CBE on the concentration of the substrates, 4MU-Glc or GC, in incubation mixtures containing homogeneous acid β-glucosidase. These results and similar data (9) obtained with CBE and δ-gluconolactone, a competitive inhibitor, provide evidence for the specific binding of Br-CBE

Figure 4



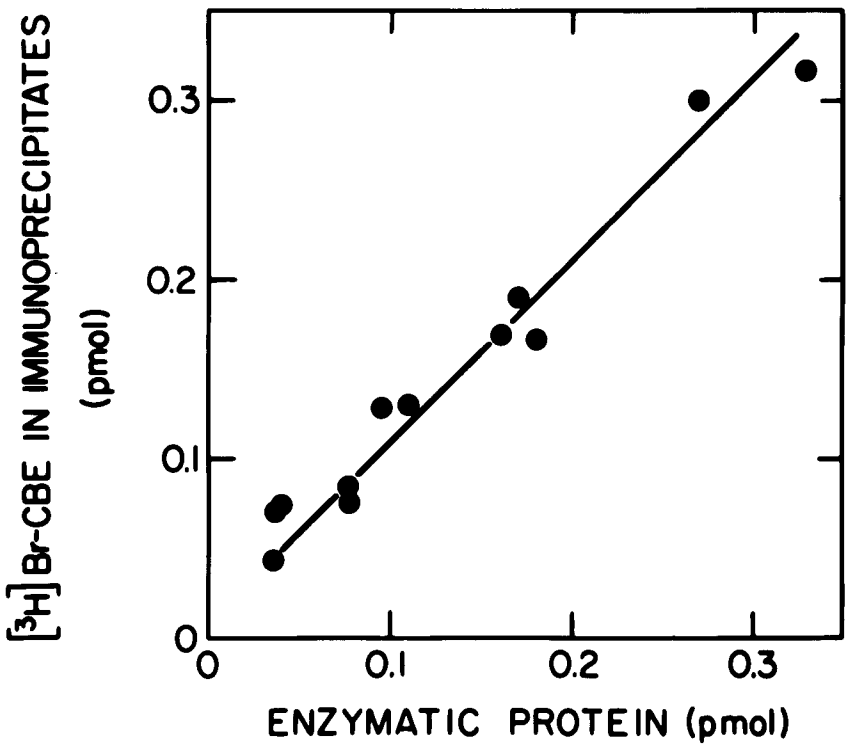
The dependence of the I_{50} values for Br-CBE on the concentrations of The substrates, 4MU-Glc (●) or GC (▲) in the incubation mixtures. Homogeneous acid β-glucosidase from placentae was the enzyme source and incubations were terminated after 1 hr.

to a catalytic site of the enzyme. To establish the number of [^{3}H]Br-CBE binding sites on acid β-glucosidase, known amounts (pmol) of enzyme were inactivated completely by a 1500- to 2500-fold excess of [^{3}H]Br-CBE (10 μM) and then separated from the unreacted inhibitor by quantitative immunoprecipitation. The mole quantity of tritium in the washed immunoprecipitates was correlated with that of pure enzymatic protein. Figure 5 demonstrates the 1:1 (mol/ mol) stoichiometry of homogeneous enzymatic protein and bound [^{3}H]Br-CBE, indicating the presence of a single catalytic site per enzyme subunit. Covalent binding of [^{3}H]Br-CBE to acid β-glucosidase was suggested by the co-elution of the enzymatic protein and radioactivity on reverse phase HPLC (Fig. 6). For these experiments, the enzyme and [^{3}H]Br-CBE (1:10; mol/mol) were incubated for 96 hr at 22°C. Under these conditions, about 13% of the enzymatic protein was labeled by [^{3}H]Br-CBE. Rechromatography of the labeled enzyme on reverse phase HPLC resulted in about a 25% loss of radioactivity associated with the protein. Autoradiographs of the SDS-PAGE gel of the pure enzyme following labeling with [^{3}H]Br-CBE demonstrated a single labeled protein species at $M_r \cong$ 67,000 (Fig. 6, inset). The finding of a single N-terminal amino acid sequence (24) and a single protein species on reverse phase HPLC (Fig. 6) or SDS-PAGE (data not shown) provided evidence for the homogeneity of the acid β-glucosidase preparations.

Isolation and Amino Acid Sequence of a Peptide Containing the Catalytic Site:

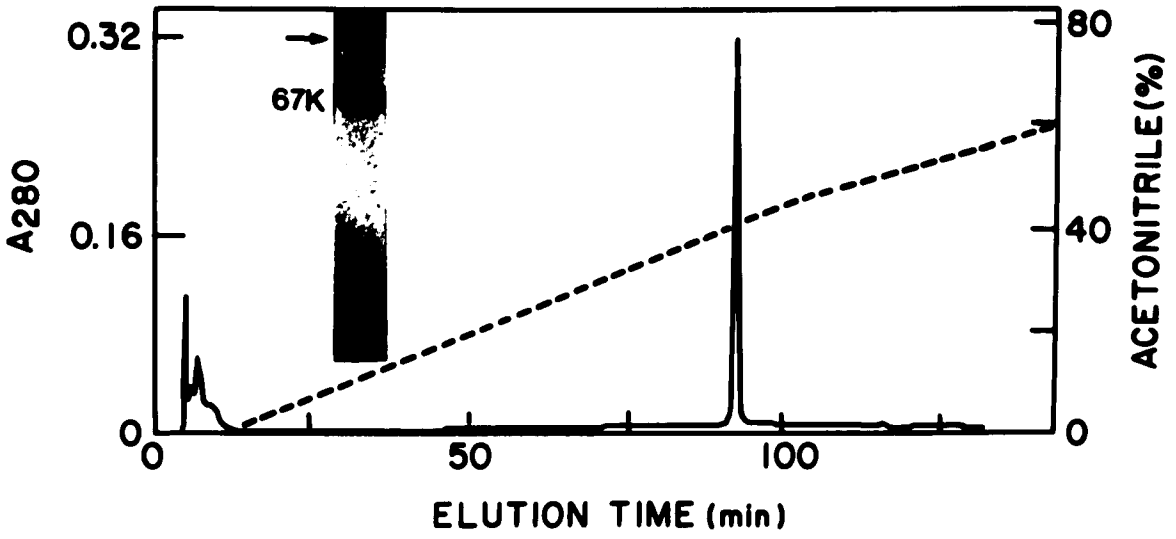
After labeling the homogeneous acid β-glucosidase with [^{3}H]Br-CBE and then digestion with V_8 protease, the radiolabeled peptides were

Figure 5



The relationship of homogeneous acid β-glucosidase protein (pmol) to [^{3}H]Br-CBE (pmol) following complete enzyme inactivation by the inhibitor. Radioactivity bound specifically to the enzyme was determined after quantitative immunoprecipitation using rabbit anti-acid β-glucosidase IgG. Unweighted linear regression analysis by the least squares method gave a slope of 0.984 with a correlation coefficient of 0.988.

Figure 6

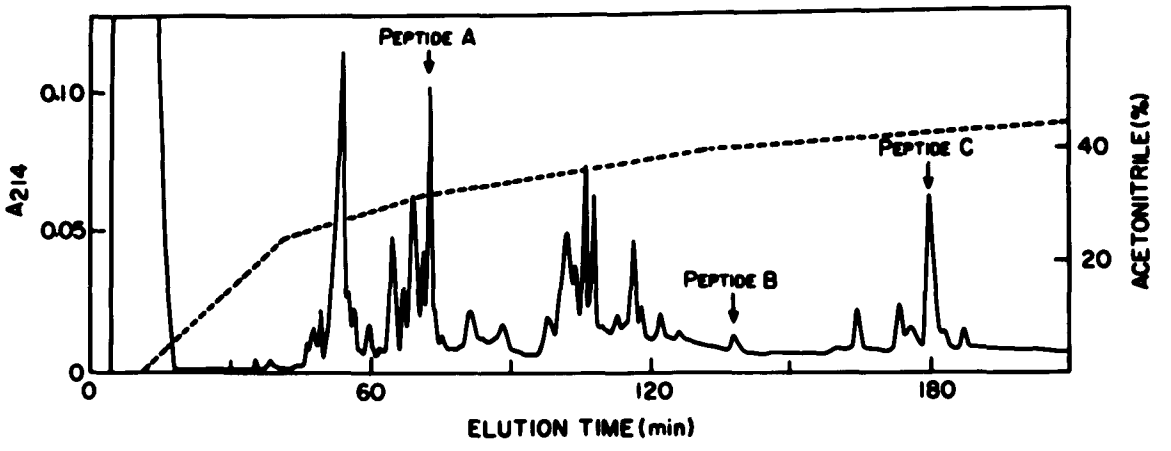


Reverse phase (Protein C-4 column, 4.6 x 250 mm) HPLC elution profile of 5.4 nmol of purified acid β-glucosidase. The pure enzyme eluted at 41.0% acetonitrile. The peaks at 6 to 10 min were absorbing compounds in the application buffer. The inset is an autoradiograph of the [^{3}H]Br-CBE labeled enzyme ($M_r \cong 67{,}000$) after SDS-PAGE (12.5%).

resolved by reverse phase HPLC using programmed nonlinear acetonitrile gradients. A typical HPLC profile of the digests is shown in Figure 7. Three peptides, designated Peptide A, B or C, which eluted at about 32%, 40% or 42% acetonitrile, respectively, contained the tritium label. To obtain optimal resolution of the labeled and unlabeled peptides, only 1 to 2 nmol aliquots of the digested, labeled enzyme were subjected to HPLC. This approach was necessary since chromatography of the isolated peptides on HPLC resulted in large losses of the tritiated label from these peptides. In addition, short exposures (1 to 2 min) of the purified labeled peptides to neutral or basic buffers containing amines or urea also resulted in loss of the radiolabel. Furthermore, the radioactive label was released during cleavage of the enzyme by cyanogen bromide using several different procedures (17-19,32). Incubation of Peptide A with trypsin in a variety of buffers did not result in cleavage.

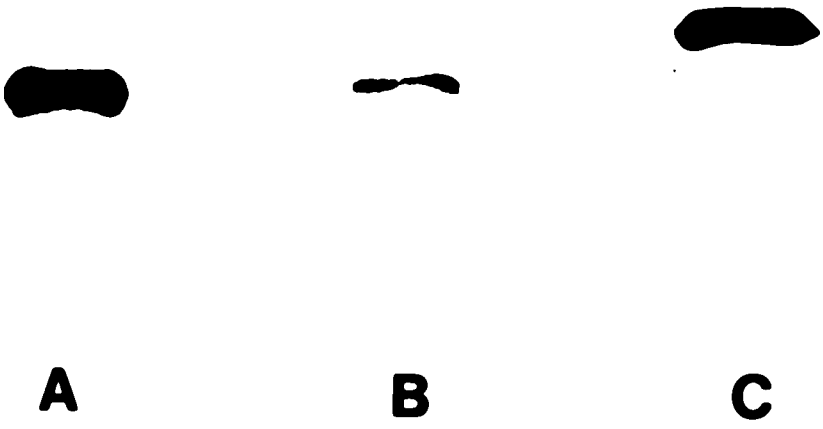
Figure 8 shows that a single protein species was present in each of the isolated labeled peptides when subjected to 12.5% SDS-PAGE. Based on the estimated labeling (8-15%) of the purified enzyme by [^{3}H]-Br-CBE (Fig. 6), about 60-75% recovery of tritium was obtained in Peptides A, B and C. Although the HPLC profiles of the peptides in the enzyme digests were somewhat variable, the principal changes were in the relative amounts of each labeled peptides; Peptide A always was present in the greatest amount. Since a 1:1 stoichiometry of enzymatic protein and [^{3}H]Br-CBE was obtained under full labeling conditions (Fig. 5), Peptides B and C probably represent overlapping sequences with Peptide A. However, the amino acid sequences of Peptides B and C could not be determined since the former was N-terminally blocked and

Figure 7



Typical reverse phase (Protein C-4 column, 4.6 x 250 mm) HPLC elution profile of 1.5 nmol of a V_8 protease digest of [^{3}H]Br-CBE-labeled acid β-glucosidase. Tritium-labeled peptides, designated Peptide A, B or C, eluted at 32%, 40% and 42% acetonitrile, respectively. The acetonitrile (ACN) gradient in 0.05% trifluoroacetic acid was 0 to 24% ACN from 12-42 min, 24 to 32% ACN from 42 to 72 min, 32 to 40% ACN from 72 to 132 min, and 40 to 45% ACN from 132 to 222 min.

Figure 8



SDS-PAGE of radiolabeled Peptides A ($M_r \cong 5,000$), B ($M_r \cong 4,500$), and C ($M_r \cong 9,500$) obtained from reverse phase HPLC.

the latter was obtained only in small amounts. Since one preparation of Peptide A also was N-terminally blocked, it was likely that the N-terminal blocking of Peptide B occurred during the cleavage and isolation procedure.

The amino acid sequence (37 residues) obtained from two different preparations of [^{3}H]Br-CBE labeled Peptide A ($M_r \cong 5000$) is shown in Figure 9. Tentative assignments are shown in parentheses and X denotes an unidentifiable amino acid residue. Peptide A contained one methionine residue and about 50% of the amino acids were hydrophobic. This sequence had exact homology to amino acid residues 429 to 465 predicted from a cDNA for human acid β-glucosidase (20), except for the second residue which was predicted to be a serine (Figure 9).

The precise amino acid to which [^{3}H]Br-CBE was bound (i.e., the catalytic site) was not identified with certainty, since the ester linkage to the peptide was labile in the presence of dimethylamine gas used during microsequencing: In separate experiments, we showed that trimethylamine liberates all tritium from Peptide A. Since it is likely that [^{3}H]Br-CBE binds to an acidic amino acid (16-18), three possible sites were indicated in this portion of Peptide A: Asp^{14}, Asp^{16} and Asp^{24}. Preliminary studies using solid-phase sequencing of Peptide A indicated the presence of radioactivity only at Asp^{14}. However, only 0.5% of the total radioactivity bound to the polystyrene beads was recovered after the sequencing procedure. Thus, confirmation of this finding will be required to assign the catalytic site to Asp^{14} with certainty.

The calculated hydropathy indices and predicted secondary structure for the sequenced portion of Peptide A are shown in Figure 10.

Figure 9

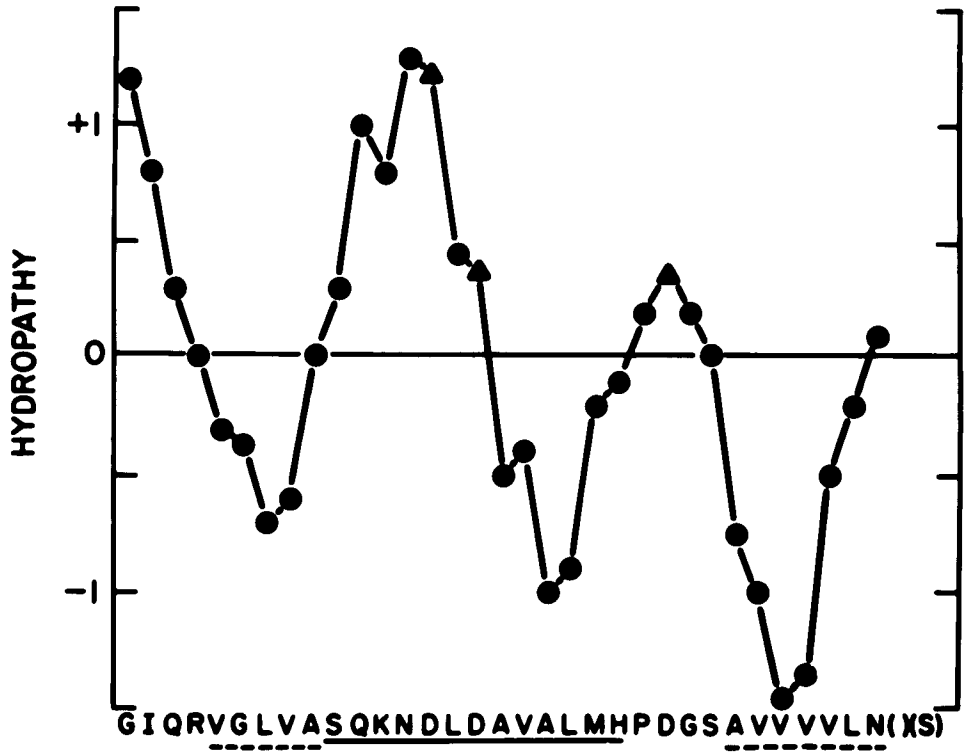
	1				5				10				15			
Peptide A	Gly	Ile	Gln	Arg	Val	Gly	Leu	Val	Ala	Ser	Gln	Lys	Asn	Asp	Leu	Asp	Ala	Val	Ala
Predicted	Gly	Ser	Gln	Arg	Val	Gly	Leu	Val	Ala	Ser	Gln	Lys	Asn	Asp	Leu	Asp	Ala	Val	Ala
cDNA	GGC	TCC	CAG	AGA	GTG	GGG	CTG	GTT	GCC	AGT	CAG	AAG	AAC	GAC	CTG	GAC	GCA	GTG	GCA

	20				25				30					35				
Peptide A	Leu	Met	His	Pro	Asp	Gly	X	(Ala)	Val	Val	Val	Val	Leu	Asn	Arg	(Ser)	X	(Lys)
Predicted	Leu	Met	His	Pro	Asp	Gly	Ser	Ala	Val	Val	Val	Val	Leu	Asn	Arg	Ser	Ser	Lys
cDNA	CTG	ATG	CAT	CCC	GAT	GGC	TCT	GCT	GTT	GTG	GTC	GTG	CTA	AAC	CGC	TCC	TCT	AAG

	40				45				50			
Predicted	Asp	Val	Pro	Pro	Thr	Ile	Lys	Asp	Pro	Ala	Val	Gly	Phe	Leu	Glu
cDNA	GAT	GTG	CCT	CCT	ACC	ATC	AAG	GAT	CCT	GCT	GTG	GGC	TTC	CTG	GAG

Colinearity of the Peptide A amino acid sequence with that predicted from the nucleotide sequence of a cDNA encoding human acid β-Glucosidase. The nucleotide sequence was from ref. 20.

Figure 10



Hydropathy profile (●) and the predicted secondary structure (---, β-pleated sheats; —— α-helixes shown below) of Peptide A which contained the catalytic site of acid β-glucosidase. Asp^{14}, Asp^{16} and Asp^{24} (▲) are possible [^{3}H]Br-CBE binding sites with Asp^{14} as the most likely candidate for the catalytic site.

Interestingly, Asp^{14} and Asp^{24} are in relatively hydrophilic areas surrounded by two hydrophobic areas. Furthermore, Asp^{14} as well as Asp^{16} appear to be in an α-helical domain, while the residues surrounding Asp^{24} may be β-pleated sheets. The tentative assignments of Asn-Arg-(Ser), residues 33 to 35, at the carboxy terminus of Peptide A indicated the presence of a site for N-glycosylation. This sequence was shown to be consistent with the predicted amino acid residues encoded by the nucleotide sequence of the acid β-glucosidase cDNA (Fig. 9). Compared to Peptide A which contained 50% hydrophobic amino acids, the amino acid composition of Peptide B ($M_r \simeq 4500$; data not shown), indicated only 31% hydrophobic amino acid residues. However, Peptide B required an 8% higher acetonitrile concentration for elution from the reverse phase HPLC than the more hydrophobic Peptide A. This finding suggested that Peptide A was glycosylated and, therefore, eluted anomalously.

Discussion

Previous studies of human acid β-glucosidase have provided information concerning physical and kinetic properties (6,8-13) and processing (14) of this lysosomal hydrolase in normal and Gaucher disease tissues. However, until the recent report of a complete cDNA sequence (20), little structural information had been available for the normal acid β-glucosidase. Therefore, the studies reported here were designed to characterize the primary structure of the human acid β-glucosidase catalytic site. These investigations were undertaken as a basis for subsequent structural analyses to correlate the kinetic abnormalities

previously identified in the defective acid β-glucosidases in unrelated patients with the subtypes and variants of Gaucher disease (8,11,12). Toward this goal, homogeneous acid β-glucosidase was reacted with [^{3}H]Br-CBE to form an ester linkage at the catalytic site (23). The labeling was specific (Fig. 4) and the interaction was solely at the catalytic site in a 1:1 mole ratio with enzymatic protein and [^{3}H]Br-CBE (Fig. 5). Amino acid sequencing of Peptide A provided the primary structure of this peptide that contained the catalytic site, but the lability of the ester bond between the inhibitor and the peptide did not permit the certain identification of the amino acid to which [^{3}H]Br-CBE was bound. The amino acid sequence data was corroborated by the colinearity with the predicted sequence for residues 429 to 465 from the acid β-glucosidase cDNA (20). Indeed, independent confirmation of the authenticity of the cDNA clone encoding acid β-glucosidase was based, in part, on the homology of the predicted amino acid sequence and that of Peptide A.

Peptide A was located near the carboxy terminus of the amino acid sequence coded by a full-length (496 amino acids) cDNA (20). The amino acid sequence of Peptide A had no significant homology with any other protein, including V_8 protease (32), in the current SEARCH program data base (>2900 entries). Also, no obvious amino acid sequence homology exists between the catalytic site sequences of fungal or almond β-glucosidases (18,19,34) and that for the human enzyme. This lack of homology was consistent with the functional properties of the plant β-glucosiases, since the almond enzyme did not cleave GC (unpublished observation).

The primary structure of the peptide containing the catalytic

site has several interesting features (Fig. 10) which may relate to the kinetic properties of the enzyme. The sequenced portion of the Peptide A contained three acidic amino acids, all of which were aspartates. By composition analysis and comparison to the predicted amino acid sequence (Fig. 9), two additional acidic amino acid residues, aspartates, were in the unsequenced portion of Peptide A. Since CBE has been shown to bind aspartate residues in the catalytic site of glucosidases from all species studied (15,18,33,34), one of the aspartates most likely binds Br-CBE. Although the lability of the ester bond during gas-phase sequencing prevented the identification of the precise binding site, preliminary studies suggest that Asp^{14} is the catalytic site. It is interesting to speculate that the Br-CBE binding site and its surrounding residues may be the structural equivalents of the third domain or "allosteric" site which have been defined by kinetic studies (9,10). Since the properties of the third domain (9) suggest the presence of an anionic residue and a surrounding hydrophobic region, each of the aspartates at residues 14, 16 or 24 of Peptide A had the necessary structure. In comparison, Asp^{38} and Asp^{45} were surrounded by more hydrophilic regions. In addition, a distinct hydrophobic domain, an aglycon binding site, for interaction with acyl chains of GC (9) has been proposed. The calculated hydropathy profile from the partial sequence of Peptide A (Fig. 10) suggests that each of the three aspartates at residues 14, 16 or 24 had the required surrounding hydrophobic structure. In particular, Asp^{14} was predicted to be in a hydrophilic pocket flanked by two hydrophobic areas, conforming closely to the kinetic model which predicted that the catalytic site would be in proximity to the hydrophobic aglycon binding site and

the third domain (8,9). Additional modeling studies will be required, once the Br-CBE binding site has been confirmed as Asp^{14}, for accurate predictions of the catalytic site's three-dimensional conformation. However, initial calculations suggest a high probability of α-helix formation (Fig. 10) in the region spanning amino acids 10 to 22 of Peptide A. The presence of a N-glycosylation site, Asn-Arg-Ser, assigned to residues 33 to 35 of Peptide A and the anomolous elution of this peptide suggested that glycosylation also may be important for proper active site conformation and, possibly, enzymatic activity (17).

In summary, these studies represent the first isolation and characterization of a catalytic site from a human lysosomal glycosidase. These results also provide a baseline for similar approaches to define the molecular basis of the abnormal acid β-glucosidase function in human Gaucher disease (11,12). Extension of these studies to elucidating the structure of the active sites of other lysosomal hydrolases may provide insight into the evolutionary relationships of these enzymes.

Chapter Four

Gaucher Disease Types 1, 2 and 3: Differential Mutations of the Acid β-Glucosidase Active Site Identified with Conduritol B Epoxide Derivatives and Sphingosine

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Materials and Methods

Reagents

The following chemicals were purchased from commercial sources: 4-methylumbelliferyl β-D-glucopyranoside (4MU-Glc; Research Products International, Mount Prospect, IL); human serum albumin (Sigma, St. Louis, MO), and Triton X-100 (Fisher Scientific, Fairfield, NJ); sodium taurocholate (Calbiochem, La Jolla, CA). All other reagents were of reagent grade or better.

Patient Population and Cell Lines

Cultured fibroblast lines were from twenty-two unrelated GD Type 1, three GD Type 2 [one female and two unrelated cell lines GM 1260 and GM 0877 (NIGMS Mutant Cell Repository; Camden, NJ)] and three Norbottnian Swedish GD Type 3 patients (provided by Dr. Lars Svennerholm). The thirteen Ashkenazi Jewish GD Type 1 patients ranged in age from 2 to 62 years at the time of diagnosis and had mild to severe clinical manifestations as assessed by the extent of hepatic, splenic, hematologic and orthopaedic involvement. The GD Type 1 patients from other ethnic backgrounds are cited below, followed in parentheses by the appropriate reference or their age at onset and major clinical findings: Hispanic (23; 4 yrs), Indian (23 yrs; hepatosplenomegaly, femoral head collapse), African Black (24, Case 26; 57 yrs), American Black (2 yrs, died at age 3 yrs; massive hepatosplenomegaly), Non-Jewish Caucasian (4 yrs, moderate hepatosplenomegaly), Cape Colored [mixed ancestry (Caucasian-African Black-Malay-Xhoisan); 25 yrs (II), 32 (III) yrs; hepatosplenomegaly, various boney manifestations],

Peruvian Indian (3 yrs; splenomegaly) and Afrikaner (25, Case 7; 21 yrs). The absence of neuronopathic disease was demonstrated in each GD Type 1 patient by normal comprehensive neurologic and developmental exams and CT scan and/or electroencephalographic studies. Eight normal control lines were established from healthy individuals of multi-ethnic backgrounds including: Caucasian, Black, Hispanic and Cape Colored.

Enzyme Sources

Fibroblast cultures were established from forearm skin explants and grown in RPMI 1640 medium containing 10% fetal calf serum. For in vitro studies, the cells were harvested at early confluency by scraping with a rubber policemen, washed twice in saline by centrifugation (1,000 x g, 10 min) and stored (-20°C) as dry pellets until used.

Enzyme Assays

Fibroblasts were disrupted and assayed for β-Glc activity with 4MU-Glc or glucosyl ceramide as described [26]. The standard incubation mixture in a total volume of 0.1 ml contained 0.04 M citrate/0.05 M sodium phosphate, pH 5.5, 4.65 mM sodium taurocholate, 4 mM Triton X-100, 50 µl of fibroblast extract, substrate and inhibitor(s). The K_m values (4MU-Glc or glucosyl ceramide) were normal for all patients.

In Vitro CBE Studies

For the determination of I_{50} values (i.e., the concentration of CBE or bromo-CBE which resulted in 50% inhibition), β-Glc activities in the normal and GD fibroblast extracts were equalized by dilution in 0.9% NaCl. Then, CBE or bromo-CBE, dissolved in water, was added to

the incubation mixture to achieve the required final concentration and the reaction was initiated by immediately adding 4MU-Glc (4 mM final concentration) or glucosyl ceramide (0.3 mM). The reactions were terminated after 2 h, and the fluorescence intensity determined [26]. For mixture experiments, equal amounts of enzymatic activity (as 1:1, v/v) from different fibroblast extracts were combined and assayed as described above. I_{50} values were determined from semi-log plots which were evaluated by the method of least squares. In the text, the I_{50} values within each group represent the mean value ± 2 SD. Preliminary studies using the natural substrate, glucosyl ceramide, with normal and GD fibroblast extracts and in highly purified β-Glc preparations (3000-fold) from spleens of some of these patients provided essentially identical data to those reported here for the 4MU-Glc substrate.

For determination of the $t_{1/2}$ values, β-Glc activities in the normal and GD fibroblast extracts were equalized by dilution in 0.04 M citrate/0.05 M phosphate, pH 5.5 containing 4.63 mM taurocholate and 4 mM Triton X-100. CBE, dissolved in the above buffer, was added to the diluted enzyme to obtain the desired final concentration. The CBE/enzyme mixtures were preincubated at 23°C for the specified time and then, aliquots were diluted to a final CBE concentration of 1 μM; preincubation at 37°C did not effect the results. The enzymatic activities in these aliquots were then determined at 37°C for 4 h. Because the enzymes in normal and, particularly, the GD fibroblast extracts were somewhat labile under the above conditions (5 to 15% loss of activity at 23°C for 2 h), a control curve for each data point was determined as follows: after the samples were incubated at 23°C without CBE for the specified time, the required amount of CBE was

added to the tubes, the mixtures immediately diluted to achieve 1 μM CBE concentration and then the enzymatic activity was determined at 37°C for 4 h. The $t_{1/2}$ values for CBE inhibition of the enzymes were determined by subtraction of the inhibition curve from the control curve. Using the above system the assays were linear with time from 1 to 6 h.

Ex Vivo Inhibition Studies

For studies of cells in culture, fibroblasts at early confluency were harvested by trypsinization for 5 min and aliquots of the cell suspension were transferred to 35 x 14 mm Petri dishes after washing two times in RPMI 1640 containing 10% fetal calf serum. After 2 to 3 days, the culture wells were washed with RPMI 1640 and the attached cells were then cultured in RPMI 1640 containing specified concentrations of bromo-CBE (added in sterile saline). After the designated amount of time (0 to 60 min), the cells were washed in fresh media without bromo-CBE and immediately harvested by trypsinization. The cells were washed with 1 ml of RPMI 1640 containing 15% fetal calf serum by centrifugation (100 x g, 10 min) and, then, washed three times with saline. The cell pellets were resuspended in 0.5 ml saline and aliquots of the extracts were assayed immediately [26].

Inhibition Studies with Sphingosine

d,l-sphingosine was prepared as described [27] and stored at -20°C in chloroform:methanol (2:1; v/v) until used. The required amount of sphingosine was aliquoted into tubes, the solvents evaporated under nitrogen and, then, the dried residue placed under high vacuum for 4 h. The dried sphingosine was resolubilized (final concentrations 0 to 300 μM) by vigorous agitation in 0.04 M cit-

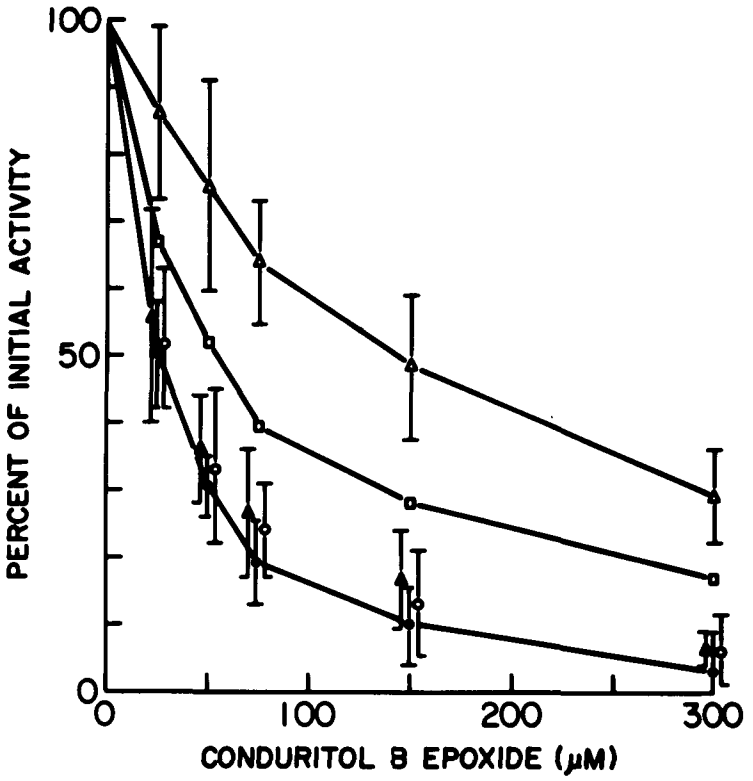
rate/0.05 M phosphate, pH 5.5 containing 4 mM Triton X-100 and 4.65 mM taurocholate. The enzyme was added, the reactions initated by adding 4MU-Glc (4 mM final concentration), and the enzymatic activity was determined as described [26]. For some experiments, CBE was added immediately prior to addition of the substrate.

Results

Inhibition of β-Glc by CBE and Bromo-CBE In Vitro

CBE inhibition curves for the normal and GD Types 1, 2 and 3 fibroblast β-Glc are shown in Figure 11. The I_{50} values for CBE or bromo-CBE for the residual β-Glc activities of the 28 GD patients and eight normal individuals are shown in Table 4. The I_{50} values for the normal fibroblast β-Glc were 28 ± 6 μM and 0.60 ± 0.09 μM for CBE and bromo-CBE, respectively. The I_{50} values obtained with the residual activities from six of the non-Jewish GD Type 1 patients [37 ± 10 μM (CBE) and 0.64 ± 0.12 μM (bromo-CBE)] and the six GD Type 2 and 3 patients [41 ± 11 μM (CBE) and 0.62 ± 0.12 μM (bromo-CBE)] were somewhat higher than the normal values but were not statistically different. In contrast, about 5-fold increased I_{50} values were obtained with the residual activities from the thirteen Ashkenazi GD Type 1 patients: 141 ± 37 μM with CBE and 3.0 ± 0.32 μM with bromo-CBE. The residual enzyme from the Cape Colored II and the Peruvian Indian GD Type 1 patients had similarly increased I_{50} values with CBE or bromo-CBE (Table 4). The residual activities from the two unrelated Cape Colored III and II GD Type 1 patients gave I_{50} values which segregated into the normal (III) or the Ashkenazi Jewish GD Type 1

Figure 11



Conduritol B epoxide inhibition of β-Glc in cultured fibroblast extracts from normal individuals (●) and GD Types 1, 2 and 3 patients. Group A residual activities were from most non-Jewish GD Type 1 (▲) and GD Types 2 and 3 (○). Group B residual activities (△) were from thirteen Ashkenazi Jewish GD Type 1 and the Cape Colored II and Peruvian Indian GD Type 1 patients. The Group C residual activity (□) was from an Afrikaner GD Type 1 patient. The bars represent ± 2 SD.

TABLE 4

CBE and Bromo-CBE Inhibition of Fibroblast β-Glucosidase Activity from Normal and GD Individuals

Source	I_{50} Value	
	CBE	Bromo-CBE
	(μM)	
Normal		
mean	28	0.60
(range)	(25-33)	(0.50-0.73)
n	8	8
Type 1 GD		
Group A		
Hispanic	30	0.64
Indian	38	0.67
African Black	36	0.56
American Black	45	0.70
Non-Jewish Caucasian	41	0.71
Cape Colored III	34	0.70
Group B		
Ashkenazi Jewish		
mean	141	3.00
(range)	(115-180)	(2.67-3.70)
n	13	13
Cape Colored II	148	3.2
Peruvian Indian	175	2.9
Group C		
Afrikaner	61	1.4
Type 2 and 3 GD		
mean	41	0.62
(range)	(32-50)	(0.52-0.69)
n	6	6

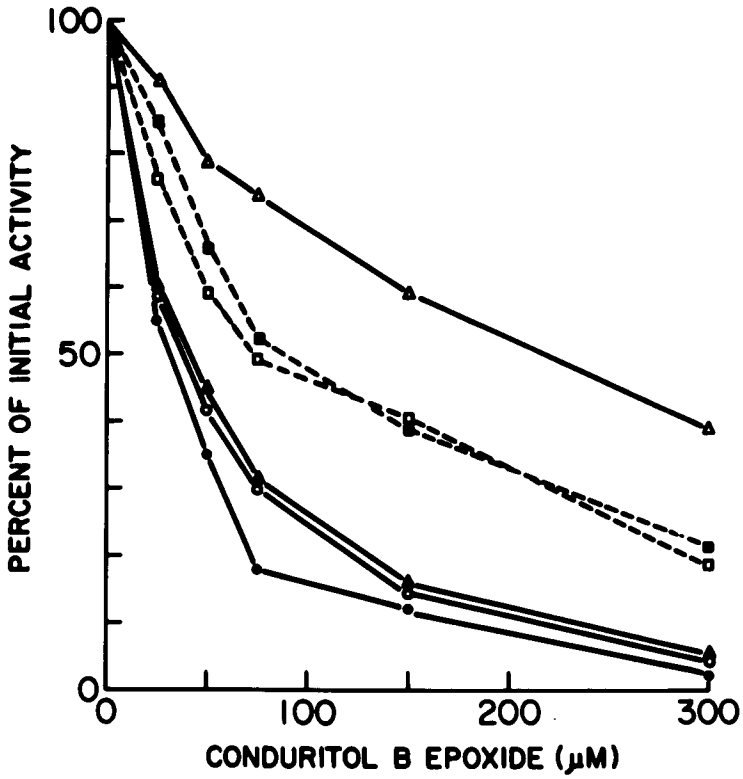
ranges (II) (Table 4). An I_{50} value intermediate between that for the normal enzyme and the Ashkenazi Jewish GD Type 1 residual β-Glc was obtained with CBE (61 μM) or bromo-CBE (1.4 μM) in the one Afrikaner GD Type 1 patient.

Based on the normal, markedly abnormal or intermediate I_{50} values, the GD residual enzyme activities were classified as Groups A, B or C, respectively. This scheme would classify the majority of non-Jewish GD Type 1 patients as well as the GD Type 2 and 3 patients as Group A. All Ashkenazi Jewish GD Type 1 patients were in Group B while the one Afrikaner patient was designated Group C. It should be noted that all Group B patients were primarily Ashkenazi Jewish and had non-neuronopathic (Type 1) GD.

Typical results of mixing experiments with equal β-Glc activities from normal and different Type 1 or 2 patients are shown in Figure 12. The mixtures of normal and Ashkenazi GD Type 1 β-Glc activities resulted in the expected intermediate CBE inhibition curve for a mixture of two enzymes with different I_{50} values (Fig. 12). Similar intermediate inhibition curves were observed for mixtures of equal activities of the GD Types 1, 2 or 3 Group A residual enzymes and that from GD Type 1 Group B (Fig. 12). Mixtures of equal amounts of β-Glc activity from normal and GD Type 1 Group A, or GD Types 2 or 3 Group A did not alter the CBE inhibition curves.

The $t_{1/2}$ values were determined for CBE inhibition of β-Glc from normal and selected GD fibroblast extracts. At 40 μM CBE the $t_{1/2}$ value for the normal β-Glc was 54 min (range = 50-65). In contrast, GD Type 1 Group B enzyme (primarily Ashkenazi Jewish) had $t_{1/2}$ values of about 218 min (range = 175-240), greater than 4-fold that for the

Figure 12



Conduritol B epoxide inhibition of mixtures of equal amounts of β-Glc activity from normal (●), Group A (non-Jewish GD Type 1, ▲) or Group B (Ashkenazi Jewish GD Type 1, Δ). Mixture of normal and Group B (■ -- ■), Group A and Group B (□ -- □), and normal and Group A (0 - 0).

normal enzyme. The GD Type 1 Group A enzymes had $t_{1/2}$ values about one and one half times of normal values (97.3; range 93-102) and the Gaucher Type 2 enzymes had normal $t_{1/2}$ values. An increased $t_{1/2}$ value of GD Type 1 Group B enzymes also was observed at 10 and 100 μM CBE. For example, at 100 μM CBE, the $t_{1/2}$ of the normal β-Glc was about 23 min [range (n = 3) = 21-25] whereas that for three Ashkenazi Jewish GD Type 1 residual activites was about 69 min [range (n = 3) = 63-75]. However, the apparent differences in $t_{1/2}$ values between the GD Type 1 Group A enzymes observed at 40 μM CBE was not found at 100 μM CBE and differences were not reproducible at 10 μM CBE.

Ex Vivo Inhibition Studies

Differential inhibition of β-Glc by bromo-CBE also was observed with viable fibroblasts in culture from normal individuals and GD Type 1 Group B patients. The degree of enzyme inhibition was dependent on time of cell exposure to bromo-CBE and the concentration of inhibitor in the media. For example, 50% inhibition of the normal enzyme was obtained at 15 or 30 min with 0.4 or 0.2 μM bromo-CBE, respectively. In contrast, 50% inhibition of the GD Type 1 Group B enzyme was achieved at about 30 or 55 min with 1.6 or 0.8 μM bromo-CBE, respectively. The I_{50} values obtained for the normal, GD Type 1 Group A, GD Types 2 and 3 and the GD Type 1 Group B fibroblast β-Glc by exposure of viable cells to several different concentrations of bromo-CBE in the media for one h were about 0.2, 0.3, 0.2 and 1.0 μM, respectively. Two or three different fibroblast cultures in each group were used for these experiments. A range of 0.1 to 0.2 μM for the I_{50} values was observed within each group.

Sphingosine Inhibition of β-Glc from Normal and GD Fibroblasts

The I_{50} values for sphingosine inhibition of β-Glc from normal and selected GD fibroblast extracts are shown in Table 5. The I_{50} values for the normal, the GD Type 1 Group A and GD Types 2 or 3 Group A β-Glc were about 40 μM. In contrast, the I_{50} value for the GD Type 1 Group B enzymes (primarily Ashkenazi Jewish) was about 186 μM, five-fold greater than that for the normal or Group A β-Glc.

Inhibition of Normal β-Glc by Sphingosine and CBE

The I_{50} values for sphingosine inhibition of normal β-Glc were linearly related to the concentration of CBE in the assay mixtures. For example, varying the CBE concentration from 0 to 100 μM increased the I_{50} values for sphingosine from 38 to 180 μM. The converse experiment demonstrated that the I_{50} values for CBE increased linearly from 27 to 103 μM in the presence of 0 to 100 μM sphingosine.

Discussion

Among the phenotypes of GD, three groups of residual β-Glc were delineated by their interaction with the covalent catalytic site inhibitors, CBE and bromo-CBE [20,21] as well as sphingosine, the cationic aglycon of glucosyl sphingosine. These three groups corresponded precisely to those that we described previously [19]. In the prior studies, the three groups (designated Groups A, B and C) of residual β-Glc activities were defined by their differential responses to negatively-charged lipids and the non-competitive cationic inhibitors, glucosyl sphingosine and its N-hexyl derivative [19]. Group A residual activities were characterized by normal responses to

TABLE 5

Sphingosine Inhibition of Fibroblast β-Glucosidase Activity from Normal and GD Individuals

Source	I_{50} Value (μM)
Normal	
mean	39
(range)	(38-42)
n	5
Type 1 GD	
Group A	
Hispanic	39
Indian	38
American Black	42
Group B	
Ashkenazi Jewish	
mean	186
(range)	(170-200)
n	6
Type 2 GD	41
Type 3 GD	38

taurocholate or phosphatidylserine and normal K_i values for the non-competitive inhibitors [19]. All these Group A residual activities (non-Jewish Type 1 and Types 2 and 3) had normal I_{50} values for CBE, bromo-CBE or sphingosine. Thus, the neuronopathic and most non-Jewish non-neuronopathic forms of GD could not be distinguished by kinetic parameters. In contrast, the Group B residual β-Glc activities required greater concentrations of taurocholate or phosphatidylserine for maximal activation and had 5- to 7-fold increased K_i values for glucosyl sphingosine and its N-hexyl derivative. All these Group B residual activities had 4- to7-fold elevated I_{50} values for CBE, bromo-CBE or sphingosine. It should be noted that all Ashkenazi Jewish GD Type 1 patients (non-neuronopathic) had Group B residual activities. Furthermore, as would be expected in an ethnically heterogeneous group, Cape Colored GD Type 1 patients III and II, who were classified into Groups A or B, respectively, in our previous studies, were distinguished by their I_{50} values using the irreversible inhibitors or sphingosine.

The fact that similar findings were obtained ex vivo (with bromo-CBE) indicates that the in vitro findings likely reflect the function of these enzymes within the lysosomal environment. The consistent differences found between these groups also indicates that these findings were due to β-Glc and not a non-relevant isozyme normally present at low levels. Furthermore, these differential findings cannot be attributed to different kinetic properties of prepro- or pro-enzymes which may be present in neuronopathic GD [16,17], since the Type 2 and 3 residual activity reacted normally with these inhibitors while the "normally processed" GD Type 1 Group B

enzyme had markedly abnormal interaction with these inhibitors.

The residual activity from the Afrikaner Type 1 patient, Group C, which had intermediate responses to taurocholate or phosphatidylserine and the reversible inhibitors [19], also had intermediate I_{50} values for CBE or bromo-CBE. These results and those of the mixture experiments suggested that the fibroblasts from this patient may have contained a mixture of Group A and B residual activities. The mixture studies also indicated that the I_{50} values alone could not distinguish between genetic compounds for the Group B mutations and the neuronopathic or non-neuronopathic Group A mutations. However, pedigree studies of this Afrikaner patient indicated that this GD Type 1 patient was a genetic compound for the GD Type 1 Group B and neuronopathic Group A mutations [25].

Based on the previous data of fibroblast β-Glc [19], we proposed that the distinguishing enzymatic defects in Groups A and B were the normal active site function in the former and abnormal active site function in the latter. The present studies provide additional support for this concept. CBE binds covalently to an acidic amino acid residue (probably aspartate) within the catalytic site of β-Glc via an oxirane ring formed during proton transfer [20,21]. Presumably this process occurs at the residue(s) associated with activation of the substrate for cleavage of the β-glucose moiety, since the hydrophilic competitive inhibitors, gluconolactone [22] or deoxynojirimycin (manuscript in preparation), compete with CBE for the catalytic site of β-Glc. Thus, the abnormal I_{50} values of CBE and bromo-CBE for the Group B β-Glc would be presumed to reflect an abnormal function [interference with proton transfer ( )] of the catalytic site. Whether

the altered function of this site was due to point mutation within the catalytic site itself or at another site which modulates catalytic site function [22] cannot be directly ascertained from the present data.

However, several observations suggest that the catalytic site itself was intact and a distinct site, the third domain [22], was altered, thus resulting in abnormal modulation of catalytic site function. The fact that the K_m values with the water soluble substrate, 4MU-Glc, were normal for all Group B GD patients [19] suggested that the affinity of the β-glucosyl moiety of substrates for the catalytic site was not detectably altered. In addition, the finding that sphingosine (I_{50} = 40 μM) was as potent an inhibitor of the normal β-Glc as a third domain probe, glucosyl sphingosine [19,22,28], indicates that the β-glucosyl moiety did not play a critical role in determining the affinity of the enzyme for these inhibitors. Since comparably increased (5- to 7-fold) amounts of sphingosine, glucosyl sphingosine or N-hexyl glucosyl sphingosine were required to inhibit the Group B residual activities, the abnormal binding of the glucosylated inhibitors was probably related to their cationic sphingosyl moieties and not the β-glucosyl residue. Thus, it is possible that a point mutation (single base change) that leads to an amino acid substitution near the third domain could result in abnormal binding of sphingosine or its glucosylated derivatives. Such a point mutation occurring at a critical residue may disrupt the proton transfer process for activation of CBE or substrate activation for cleavage. Such a mutation might explain the abnormal CBE and bromo-CBE binding (Tables 4 and 5) as well as the abnormal affinity of

the Group B residual enzyme for sphingosine and its glucosylated derivatives. Also, these observations, the fact that glucosyl sphingosine is a substrate for β-Glc [29], and the finding that CBE interferes with sphingosine inhibition of β-Glc suggest that the third domain and the catalytic site, although kinetically distinct [22], are physically contiguous and that their functions are related to V_{max}, e.g. k_{cat}.

These observations and previous studies [19,27,30] are consistent with a mutation in the structural gene of β-Glc from the Ashkenazi Jewish GD Type 1 Group B patients which alters the function of the third domain, i.e., a true V_{max} mutation [30]. In contrast, the three active site domains of β-Glc (the catalytic site, the aglycon binding site and the third domain [22]) apparently function normally or nearly normally in most non-Jewish Type 1 and the Type 2 and 3 phenotypes of GD. These studies not only provide further evidence of the enzymatic heterogeneity of the Gaucher phenotypes but also provide additional methods to identify candidates for genetic studies with cDNA or genomic probes for the structural gene of β-Glc [31].

Chapter Five

The Use of Conduritol B Epoxide Derivatives to Investigate the Catalytically Active Normal and Gaucher Disease Acid β-Glucosidases

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Materials

The following were from commercial sources: Triton X-100, β-D-glucose (Sigma Chemical Co., St. Louis, MO), sodium taurocholate (Calbiochem, La Jolla, CA), sodium dodecyl sulfate (SDS, British Drug House, Poole, UK), NBD-dodecanoic and NBD-hexanoic acids (Molecular Probes, Junction City, OR) and 4-methylumbelliferyl-1-O-β-glucoside (4-C_1-U-Glc) (RPI, Mount Prospect, IL). 4-C_1-U-Glc was recrystallized from ethanol prior to use. All other reagents were used directly.

The following compounds were synthesized as described: NBD-dodecaoyl (NBD-C_{12}-)- and NBD-hexanoyl(NBD-C_6-)-GC (18), CBE (19), [^{3}H]Br-CBE (19), and 4-alkyl (C_7, C_9, C_{11}) umbelliferyl-1-O-β-glucosides (4-C_n-U-Glc) (20).

Methods

Enzyme Sources:

Normal β-Glc was purified from human placentae by affinity chromatography on N-alkyl-deoxynojirimycin-Sepharose (21). Homogeneity of the preparation was documented by the presence of a single protein species on SDS-polyacrylamide electrophoresis or reverse phase HPLC (17) and by a single N-terminal amino acid sequence (21). Normal spleen was obtained at surgery from a patient with idiopathic thrombocytopenic purpura and stored at -20°C for six months. The β-Glc from this spleen was purified through the butanol delipidation step (5) and was about 60-fold enriched. The Type 1 AJGD spleens were obtained at splenectomy from 25 and 54 yr. old patients and stored at -20°C until used (6-12

months). The mutant splenic β-Glc were purified either by hydrophobic (3) or N-alkyl-deoxynojirimycin-Sepharose chromatography (21). These final preparations were about 500- and 7500-fold enriched and several protein bands were observed on SDS-polyacrylamide gel electrophoresis. The 500-fold enriched Type 1 AJGD enzyme also was delipidated with 40% of a 1:1 (v/v) mixture of butanol: isopropyl ether to ensure that intrinsic lipids were removed (6). All enzyme preparations were stable for several months when stored at 4°C in 0.04 M citrate/0.05 phosphate, pH 5.5, 4 mM β-mercaptoethanol, and 1 mM EDTA (buffer A) containing 60-80% ethylene glycol. These four different enzyme preparations of varying purity were used in comparative studies to control for the effect of contaminants on the kinetic measurements since homogeneous Type 1 AJGD β-Glc could not be obtained in an active form.

Using the pure normal placental β-Glc and the partially purified normal or Type 1 AJGD splenic enzymes [which had been deglycosylated with N-glycanase (Genzyme, Boston, MA)], identical immunoblot molecular weights (56,000) were obtained with monospecific polyclonal or monoclonal antibodies to the normal placental β-Glc. In addition, immunoblots demonstrated identical profiles of cross-reacting immunologic material (CRIM) from the glycosylated enzymes in splenic or fibroblast extracts from several normal individuals or other Type 1 AJGD patients although the profiles in each tissue source were different( ). These studies indicated that the partially purified mutant enzymes examined had essentially identical molecular weight to the normal placental or splenic enzymes and they were similar to those from several other Type 1 AJGD patients.

Kinetic Studies:

Hydrolysis of NBD-GC derivatives (9) or 4-C_n-U-Glc (9,18) was determined fluorometrically. The typical reaction mixture (0.2 ml) contained 0.05 M phosphate/0.04 M citrate, pH 5.5, Triton X-100 (4 mM), 4.65 mM taurocholate, 4 mM β-mercaptoethanol, 1 mM EDTA, substrate, < 1% ethylene glycol, and enzyme. Assays with the highly purified Type 1 AJGD enzyme contained 0.6% human serum albumin in the incubation mixtures to maintain enzyme stability. Under these conditions, human serum albumin did not alter the k_{cat} or V_{max} values of the more stable pure normal placental or delipidated splenic β-Glc, respectively. The lipoidal substrates in chloroform/methanol (2:1; v/v) were added to dry tubes, the solvents evaporated under nitrogen and then with high vacuum for 2 to 4 h. The residue was resuspended in buffer containing Triton X-100 and the reactions were initiated by the addition of enzyme. The amount of enzyme was adjusted to ensure that less than 5% of the substrate was hydrolyzed. Reactions at 37°C were terminated after 0.5 to 2 h. Protein concentrations were estimated by the method of Lowry et al. (22).

Determination of k_{cat} Values:

The k_{cat} values for alternate substrates with the normal and Type 1 AJGD β-Glc were calculated from $V_{max} = [E]_t\ k_{cat}$ assuming that only those active sites which retained their respective full catalytic activity were labelled by [^{3}H]Br-CBE (see below). V_{max} values for the 4-C_1- and C_7-U-Glc and NBD-C_6- or NBD-C_{12}-GC substrates were obtained from linear Lineweaver-Burk plots which had been evaluated by the least squares method. The graphic method of Cleland (23) was used to deter-

mine K_m and V_{max} for the 4-C_9- and -C_{11}-U-Glc, since substrate inhibition was observed. The k_{cat} values were based on six separate experiments performed in duplicate for each substrate and enzyme source. For studies which determined k_{cat} from the relationship of the enzyme activity to the number of catalytic sites (i.e., Fig. 13), enzyme activities were determined with subsaturating amounts of substrate (4 mM 4-C_1-U-Glc or 0.2 mM NBD-C_{12}-GC) to avoid the problem of substrate insolubility at high concentrations: The V_{max} was calculated from the substrate concentrations and respective K_m values.

$[E]_t$ was estimated by quantitating the number of catalytic sites in each enzyme preparation using [^{3}H]Br-CBE (8000 cpm/pmole) as follows: various amounts of enzymatic activity or protein from the different enzyme preparations in buffer A were incubated with a large excess of [^{3}H]Br-CBE (2-8 µl; 10 µM final concentration) in 0.6% human serum albumin. Complete inactivation of each enzyme was achieved with this concentration of [^{3}H]Br-CBE by 2 h at 22°C. To ensure that all [^{3}H]Br-CBE binding sites were saturated, the mixtures were incubated at 22°C for 24 h. Human serum albumin was required to maintain enzyme stability under these conditions for up to 24 h.

To separate [^{3}H]Br-CBE from that bound specifically to β-Glc, the enzyme-[^{3}H]Br-CBE complexes were immunoprecipitated quantitatively with monospecific rabbit anti-human β-Glc IgG and Staphlococcus aureus Protein A (13). The resultant supernatants were reprecipitated successively with additional IgG and Protein A until no additional increase in precipitated radioactivity was observed; immunoprecipitation usually was quantitative after a single cycle. The resultant pellets were washed by resuspension and centrifugation (10,000 x g; 40 min)

twice in phosphate buffered saline, containing 1% human serum albumin, 0.5 M NaCl and 0.05% Tween 20 and then twice in phosphate buffered saline, containing 0.05% Tween 20. The washed pellets were dissolved (24 h, 22°C) with 100 µl Protosol™ (New England Nuclear, Boston, MA) in 900 µl of water and the radioactivity determined. With a fixed amount of the pure placental or crude splenic enzymes (<2 nmol 4-C_1-U-Glc hydrolyzed/min) from normal sources, the number of catalytic sites was constant when the concentration of [^{3}H]Br-CBE was varied between 2 and 20 µM. With similar amounts of enzymatic activity from the Type 1 AJGD enzyme preparations, the number of catalytic sites remained constant when the concentration of [^{3}H]Br-CBE was varied between from 5 and 20 µM. Based on these results, a final concentration of 10 µM [^{3}H]-Br-CBE was used in these experiments.

The concentration of catalytic sites/mg protein of original enzyme solution was determined from the radioactivity in the precipitates. The total enzyme concentration, [E_t], was based on a molecular weight of 56,000 for the pure unglycosylated normal enzyme which was calculated from amino acid composition (21). This protocol provided a 1:1 mol/mol stoichiometry of [^{3}H]Br-CBE in the precipitates and pure normal placental enzymatic protein (17).

Determination of K_i and k_{max} Values for CBE:

For the determination of K_i (dissociation constant) and k_{max} (maximal rate of inactivation) values with CBE, the normal or Type 1 AJGD enzymes in buffer A were incubated in the presence of the required amount of CBE and 0.5% each of Triton X-100 and human serum albumin (120 µl final volume) at 22°C. This temperature was used since at 37°C,

the β-Glc in the different preparations was not equally stable. At indicated times, 5 µl aliquots of these mixtures were immediately diluted with 0.04 M citrate/0.05 M phosphate, pH 5.5, containing 4 mM β-mercaptoethanol and 1 mM EDTA, to a final CBE concentration of less than 1 µM for the normal enzymes and 1-4 µM for the Type 1 AJGD enzymes. Control experiments demonstrated that the respective enzymes were stable under these conditions. An additional control included the respective enzyme incubated in the absence of CBE for the indicated time. Then the desired concentration of CBE (2.5 µl), was rapidly mixed with a 2.5 µl aliquot of the incubated enzyme and immediately diluted to the final concentrations of CBE indicated above. These controls were used as zero time points for the CBE inactivation curves of the respective enzymes: they differed from true zero time points (i.e., no CBE) by less than ±10%. Enzymatic activities were determined after incubation of the diluted enzyme-CBE mixture with 4-C_1-U-Glc (4 mM) for 2 h at 37°C (9,18).

The K_i and k_{max} of the respective enzymes for CBE were determined from the model

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} EI \xrightarrow{k_{max}} E^*I$$

where EI is a reversible complex and E*I is the inactivated enzyme with the inhibitor covalently bound. This model is described by

$$1/k_{app} = K_i/k_{max} \cdot 1/[I] + 1/k_{max} \quad (25) \qquad (1)$$

where k_{app}, the apparent inactivation rate constant, was determined at each [I], CBE concentration, by

$$\ln (E/E_0) = -k_{app}t \quad (25). \qquad (2)$$

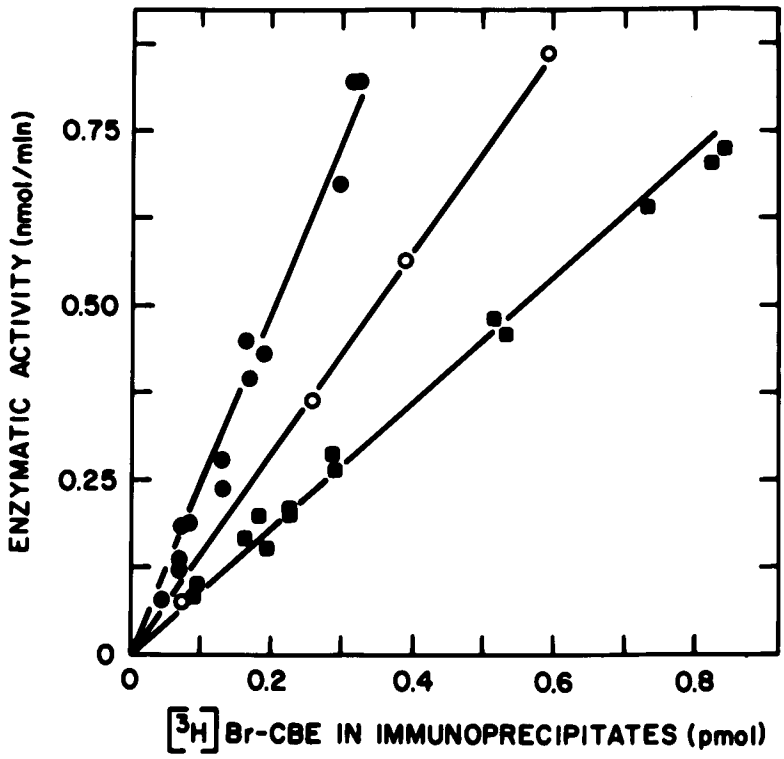
E and E_o were the remaining enzyme activity at time t and the original enzyme activity with the 4-C_1-U-Glc (4 mM) substrate, respectively (9,18). The constants in equations (1) and (2) were determined from unweighted linear regression curves evaluated by the method of least squares. The results are reported as means and ranges from five separate experiments performed in triplicate for the normal placental enzyme and three separate experiments in triplicate with the Type 1 AJGD splenic enzyme preparations.

Results

Determination of k_{cat} Values:

To determine the k_{cat} values for the normal and Type 1 AJGD active enzymes, the concentration of catalytic sites in each preparation was determined using [^{3}H]Br-CBE and monospecific rabbit anti-human β-Glc IgG. As shown in Figure 13, the amount of enzymatic activity (4-C_1-U-Glc) was directly related to the number of catalytic sites which were specifically labeled by [^{3}H]Br-CBE in the respective enzyme preparations; similar results were obtained using NBD-C_{12}-GC [200 μM; K_m = 30 μM (Table 6)] as substrate (data not shown). The k_{cat} values for the normal and Type 1 AJGD enzymes with 4-C_1-U-Glc were obtained directly from the slopes of the respective curves in Figure 13 and the corresponding data with NBD-C_{12}-GC as substrate. The k_{cat} values for the normal homogeneous placental enzyme, with the 4-C_1-U-Glc and NBD-C_{12}-GC substrates, were 2360 min^{-1} (range 2240 to 2480) and 2440 min^{-1} (range 2320 to 2560), respectively. Using the delipidated normal

Figure 13



Correlation of enzymatic activity (nmol 4-C_1-U-Glc hydrolyzed/min) and the number of catalytic sites in homogeneous normal placental β-Glc (●), delipidated normal splenic enzyme (○) and partially purified Type 1 AJGD splenic β-Glc preparations (■). [^{3}H]Br-CBE specifically bound to the respective β-Glc was determined by quantitative immunoprecipitation of the enzyme-[^{3}H]Br-CBE complex with rabbit anti-human placental β-Glc IgG and Staphlococcus Aureus protein A. The k_{cat} values for each enzyme preparation were determined from the slopes of the unweighted linear regression curves (see text and Table 6). The data points for the mutant enzyme were from two different enzyme preparations which differed in degree of purification by 15-fold.

TABLE 6

Comparison of Kinetic Constants of the Normal and Type 1 AJGD β-GLC[1] with Alternate Substrates

Substrate Aglycon	K_m(mM)		k_{cat} (min^{-1})				
			Normal Placental		Normal Splenic		AJGD Splenic
	Normal	AJGD	Method 1[3]	Method 2[4]	Method 1	Method 2	
2-N-(NBD-C_{12}-)-sphingosyl	0.03±0.01[2]	0.03±0.02	2440 (2320-2560)	2135 (2090-2178)	1480 (1410-1510)	980 (880-1080)	826 (749-893)
2-N-(NBD-C_6-)-sphingosyl	0.05±0.02	0.05±0.03		3200 (2790-3610)			1090 (963-1218)
4-C_{11}-U	0.05±0.02	0.05±0.02		773 (729-818)			306 (278-329)
4-C_9-U	0.17±0.03	0.15±0.02		2220 (1973-2460)			914 (850-989)
4-C_7-U	0.12±0.02	0.28±0.05		1972 (1820-2124)			713 (670-748)
4-C_1-U	1.9±0.3	2.9±0.5	2360 (2240-2480)	2235 (2038-2430)	1380 (1370-1390)	920 (830-1000)	893 (862-929)

Table 6 (continued)

[1] Homogeneous normal placental β-Glc and partially purified normal or Type 1 AJGD splenic enzyme were used for these studies.

[2] These results supercede previous reports in which a 10-fold arithmatic error was made in the K_m values (5,8,9,12,13).

[3] Method 1: k_{cat} was derived from the slopes of enzymatic activity as a function of [^{3}H]Br-CBE in the immunoprecipitates (see Figure 13 and text).

[4] Method 2: k_{cat} was derived from $V_{max} = [E_t]k_{cat}$ where $[E_t]$ was determined from the radioactivity of [^{3}H]Br-CBE in immunoprecipitates after complete inactivation of a fixed amount of enzyme activity by 10 μM of [^{3}H]Br-CBE (see text).

splenic extract, the k_{cat} values were 1380 min^{-1} (range 1370 to 1390) for the 4-C_1-U-Glc substrate (Fig. 13) and 1480 min^{-1} (range 1410 to 1510) with NBD-C_{12}-GC. The corresponding k_{cat} values for the Type 1 AJGD splenic enzymes were 920 min^{-1} (range 830 to 1000) with 4-C_1-U-Glc and 980 min^{-1} (range 880 to 1080) with NBD-C_{12}-GC.

In separate sets of experiments similar to those in Figure 13, the number of catalytic sites in the normal or Type 1 AJGD β-Glc sources was determined as a function of total protein concentration in the respective enzyme preparations. These data indicated that [^{3}H]Br-CBE bound to the homogeneous normal placental enzyme in a 1:1 mole ratio with enzymatic protein (17). The normal splenic β-Glc was estimated to represent about 0.4-0.6% of the total protein in the preparation. Similary, the Type 1 AJGD active enzymatic protein was 0.7% and 10.5% of the total protein in the two different preparations. From these data, the total enzyme concentrations, [E_t], were calculated to be between 0.5 and 15 nM: i.e., about 20,000- to 700-fold less than the concentration of [^{3}H]Br-CBE (10 μM) used in these experiments.

The k_{cat} values (Table 6) for the pure normal placental enzyme and the Type 1 AJGD splenic enzymes with each substrate were determined from $V_{max} = [E_t]k_{cat}$, assuming that only enzyme molecules which had retained full catalytic activity had been quantitated by the [^{3}H]Br-CBE method. For each substrate, the k_{cat} values were about 1.5- to 3-fold lower for the Type 1 AJGD enzymes than the respective values for pure normal placental β-Glc. Essentially identical k_{cat} values were obtained with either Type 1 AJGD splenic preparation. As indicated above, the k_{cat} values for 4-C_1-U-Glc and NBD-C_{12}-GC as substrates with the crude normal splenic preparation were about 1.3- to 1.7-fold

greater than those for the Type 1 AJGD splenic enzymes. The respective K_m values for each substrate were similar in the Type 1 AJGD and normal enzyme preparations (Table 6).

Determination of K_i and k_{max} Values for CBE:

In order to obtain independent confirmation that the k_{cat} values for the Type 1 AJGD enzymes were about 1.5- to 3-fold lower than the normal values, the interaction of CBE, the parent compound of [^{3}H]Br-CBE, with these enzymes was evaluated. Our previous finding that increasing concentrations of δ-gluconolactone (9), sphingosine (12), or substrates [4-C_1-U-Glc or NBD-C_{12}-GC (17)] directly increased the I_{50} values for CBE with the normal enzyme, suggested that a non-covalent intermediate was formed prior to covalent attachment of CBE to the enzyme. To evaluate this model, a series of inactivation rate curves was developed using the pure normal placental β-Glc and the Type 1 AJGD splenic enzymes (Fig. 14). The data, plotted according to equation 2, were linear, indicating a first-order process. To determined the K_i (dissociation constant) and the k_{max} (maximal rate of inactivation) values, the data in Figure 14 were replotted according to equation 1 (Fig. 15). The $1/k_{app}$ -intercept provides $1/k_{max}$ and the 1/[CBE]-intercept is $-1/K_i$. The k_{max} values for the normal placental enzyme (0.051 min^{-1}, range = 0.042 to 0.060 min^{-1}) and the Type 1 AJGD enzymes (0.058 min^{-1}, range = 0.042 to 0.074 min^{-1}) were not different. In contrast, the K_i value for the Type 1 AJGD enzymes (839 μM, range = 775 to 903) was about five times that for the normal enzyme (K_i = 166 μM, range = 109 to 233).

Using these values and equation 1, the predicted inactivation

Figure 14

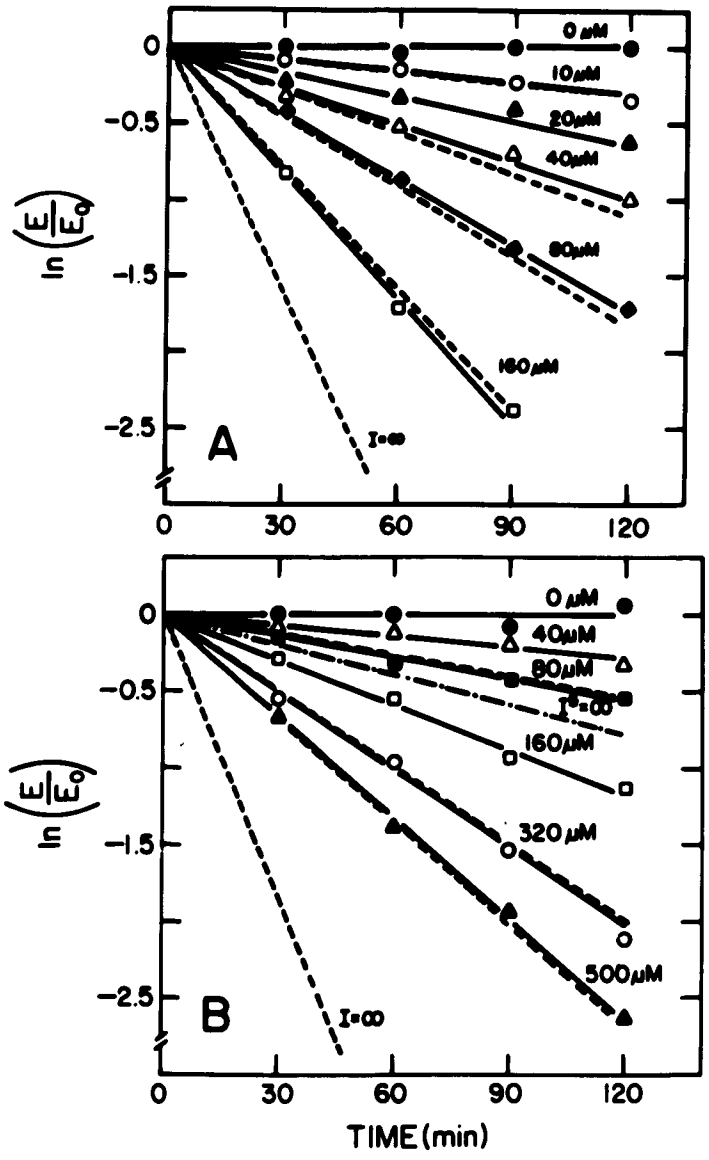
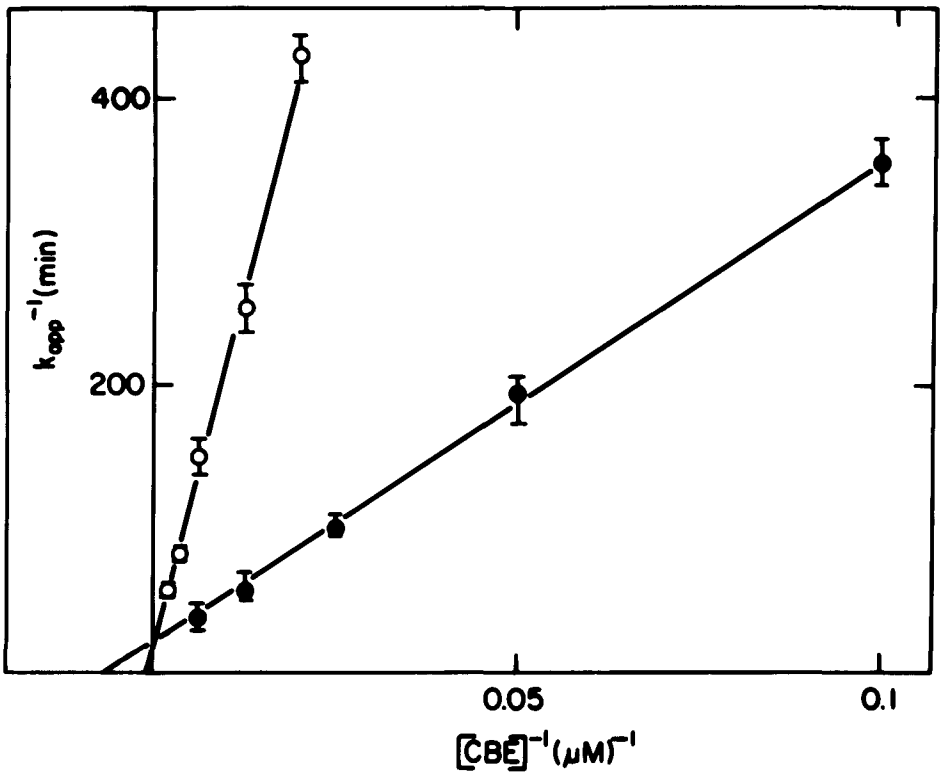

0 μM
10 μM
20 μM
40 μM
80 μM
160 μM
I=∞
A
0
-0.5
-1.5
-2.5
0
30
60
90
120
ln ω/ω°
0 μM
40 μM
80 μM
I=∞
160 μM
320 μM
500 μM
B
TIME (min)

Figure 14:

Rates of inactivation of pure normal placental β-Glc (A) and partially purified Type 1 AJGD splenic enzyme (B) by varying concentrations of CBE. The data were plotted according to equation 2 (see text) where E and E_0 represent the remaining enzymatic activity at time t and the original enzymatic activity, respectively. The theoretical curves (---) for the respective enzymes were calculated from equation 2 based on the K_i and k_{max} values obtained from Figure 15 and equation 1 (see text). The I =∞ curves were theoretical curves where $k_{app} = k_{max}$. I* in B refers to the theoretical curve at I =∞ when k_{max} for the AJGD Type 1 enzyme was one-tenth of the normal value.

Figure 15



Determination of k_{max} (maximal rate of inactivation) and K_i (dissociation constant) for CBE with the pure normal placental β-Glc (●) and the partially purified Type 1 AJGD splenic enzymes (○). The data were evaluated according to equation 1 where the k_{app}^{-1} intercepts are k_{max}^{-1} and the $[CBE]^{-1}$ intercepts are $-K_i^{-1}$.

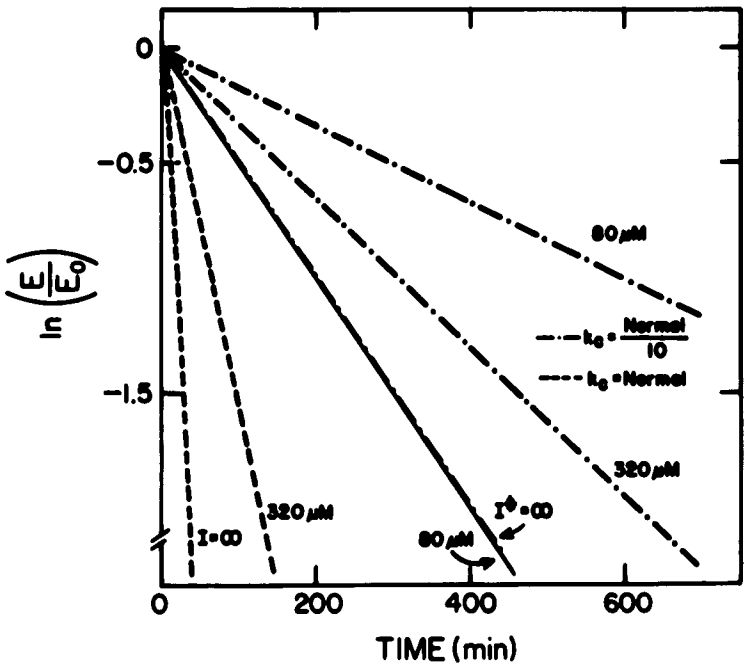
rate curves showed a close correspondence to those obtained experimentally (Fig. 14). Furthermore, the predicted inactivation rate curves at infinite [CBE] were identical for the normal and Type 1 AJGD enzymes. In comparison, if the k_{max} values for the Type 1 AJGD enzymes were 10-fold less than those for the normal enzyme, completely different inactivation rate curves would be obtained (Fig. 16). In this case, the inactivation rate curve at infinite [CBE] would correspond closely to that observed with the Type 1 AJGD enzymes at 80 μM of CBE (Fig. 16). Compared to normal β-Glc, a 1.5- to 3-fold decrease in the k_{max} value of the Type 1 AJGD enzymes would not be readily apparent. However, the close correlation of the predicted and experimental curves suggest that such a difference in k_{max} was unlikely.

The inactivation rate curves at several CBE concentrations also were used to determine the stoichiometry of binding with the normal and Type 1 AJGD enzymes (27). Figure 17 is a replot of $\log(1/t_{1/2})$ vs. log[CBE]. The slope of these Hill plots were 0.94 and 1.08 for the normal and Type 1 AJGD enzymes, respectively, indicating a 1:1 stoichiometry between CBE and either enzyme. These results confirm our previous studies of the normal enzyme based on protein concentration (17) and establish the binding relationship for these Type 1 AJGD enzymes.

Discussion

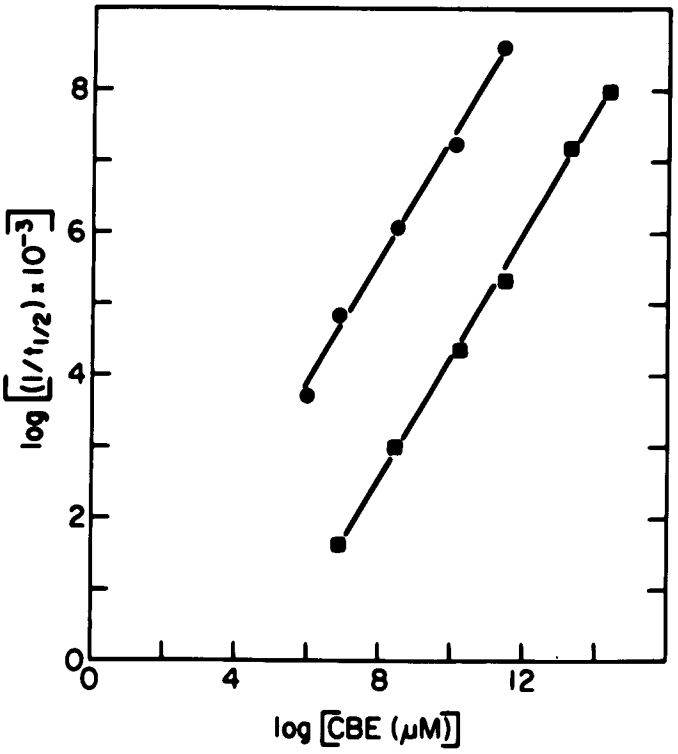
In this communication, we report studies which provide evidence for a defect of active site function of β-Glc from patients with Type 1 AJGD. Compared to the normal enzyme, the defect in the Type 1 AJGD enzyme resulted in a small decrease (1.5- to 3-fold) in the k_{cat} values

Figure 16



Theoretical curves for the rates (equations 1 and 2) of inactivation of the Type 1 AJGD enzyme assuming that 1) k_{max} was normal and K_i was 5-fold increased (---) and 2) k_{max} was 10-fold less than the normal k_{max} and K_i was normal (-·-); I* is the corresponding curve at I =∞ for this model. The first alternative conforms well to the observed inactivation rate curves (Figure 14B).

Figure 17



Replot of $\log[(1/t_{1/2})\times10^{-3}]$ value from Figures 14A and B as a function of log[CBE]. These Hill plots for the normal (●) and Type 1 AJGD (■) enzymes had slopes of 0.94 and 1.08, respectively, indicating a 1:1 stoichiometry of CBE binding to each enzyme.

for several substrates and a 5-fold decreased affinity for CBE, a covalent catalytic site inhibitor. These results imply that the deficiency of enzymatic activity in Type 1 AJGD cannot be explained by a major alteration in the catalytic capacity of the active mutant enzyme. This explanation for the nature of the defect in Type 1 AJGD differs from that previously suggested by others (15) and by us (12) which proposed that the defect in the Type 1 AJGD enzyme resulted in a large decrease in V_{max} or k_{cat}. Based on comparative immunologic studies of normal and Type 1 Gaucher disease enzymes in highly purified splenic preparations Pentchev et al. (15) suggested a mutation in the β-Glc structural gene which resulted in a 10- to 20-fold decrease in V_{max} and, by inference, k_{cat} of the Type 1 Gaucher disease enzyme. Using immunologic, inhibitor and inactivation (CBE) studies of the residual enzyme in fibroblast extracts, we proposed a specific active site defect which could account for this V_{max} abnormality in Type 1 AJGD (8). More recently, we suggestd that the abnormal <u>in vitro</u> and <u>ex vivo</u> inactivation rates of the Type 1 AJGD enzyme by CBE or Br-CBE may be due to a defective proton transfer process required for activation of the CBE oxirane ring (12). However, this latter suggestion was based on the assumption that, similar to fungal β-glucosidases, the affinity of CBE for the human enzyme was weak (i.e., $[I] \ll K_i$): The observed rate constant under these conditions would be k_{max}/K_i. Because of the nearly identical K_m or K_i values for 4-C_1-U-Glc or β-glucose (9), respectively, with the normal and Type 1 AJGD enzymes as well as the structural similarity of CBE and β-glucose, we assumed that the affinity (i.e., K_i value) of CBE for either enzyme would be the same. The observed difference in the inactivation rates, therefore, was ascribed to

a lower k_{max} and, by inference, a decreased k_{cat} for the Type 1 AJGD enzyme (12). In contrast, the present data conform well to a model that predicts that the normal or Type 1 AJGD β-Glc and CBE form reversible EI complexes prior to inactivation and that the dissociation constant (K_i) of CBE was about 1500-fold lower than that for β-glucose (K_i = 220 mM^2). These results indicate that the K_i value for CBE was abnormal in Type 1 AJGD while the k_{max} was normal.

Previous studies have been limited by the lack of homogeneous Type 1 AJGD enzyme which retained full catalytic activity to measure k_{cat} directly and by assumptions of antigenic identity of the normal and Type 1 AJGD enzymes (13,15,16). To overcome these limitations, we used the covalent inhibitor, [^{3}H]Br-CBE, to determine the k_{cat} values from V_{max} values and the concentration of β-Glc catalytic sites, [E_t], in pure normal placental β-Glc, delipidated crude normal splenic extracts, and the Type 1 AJGD enzyme preparations. This approach obviated the need for homogeneous mutant enzyme which retained full catalytic activity and only required that all enzyme which bound [^{3}H]Br-CBE could be completely immunoprecipitated. Thus, the results of these studies were dependent upon the degree of labeling obtained with the Type 1 AJGD enzyme. Complete labeling of the active Type 1 AJGD enzyme was supported by the following findings: 1) [^{3}H]Br-CBE was recovered from SDS-polyacrylamide electrophoretic gels of the pure normal enzyme and the corresponding protein band in the Type 1 AJGD enzyme preparation following immunoabsorption with monoclonal antibody to homogeneous normal β-Glc (unpublished observation). These results indicated covalent binding of [^{3}H]Br-CBE to either enzyme without large losses of label under the less stringent procedures used in the present experi-

ments. 2) Labeling conditions were optimized to ensure that all [^{3}H]Br-CBE binding sites were saturated. 3) A 1:1 mole to mole stoichiometry was obtained with the pure normal enzymatic protein and [^{3}H]Br-CBE [(17) and Fig. 17], as well as for the Type 1 AJGD enzyme (Fig. 17). 4) A linear relationship was found between the amount of incorporated radioactivity and enzymatic activity for the normal or Type 1 AJGD enzymes (Fig. 13) which had grossly similar primary structure (see methods). The calculated k_{cat} from these curves for the pure normal placental enzyme agreed well with the observed value based on protein determinations (Fig. 13 and Table 6). 5) The maximal rate of inactivation (k_{max}) of the Type 1 AJGD enzyme by CBE was similar to that for the normal placental β-Glc. This result is inconsistent with a major alteration in k_{cat}, if the steps required for covalent binding of [^{3}H]-Br-CBE and substrate hydrolysis are the same (26). In addition, we assumed that only active enzyme could bind [^{3}H]Br-CBE covalently and that these molecules had retained their respective full catalytic activities. The finding of the 1:1 stoichiometry of inhibitor binding to the normal enzyme based on protein (17) or activity determinations support this assumption. The normal k_{max} values and minor decrease in k_{cat} values for the Type 1 AJGD splenic enzymes provide evidence for the binding of [^{3}H]Br-CBE only to catalytically active mutant β-Glc. If the first assumption was incorrect or the immunoprecipitation procedure quantitated non-specifically bound (i.e., non-β-Glc) [^{3}H]Br-CBE, the differences in k_{cat} values between the normal placental β-Glc and Type 1 AJGD active enzyme would be less than those obtained.

Although the present studies were confined to splenic enzymes from two Type 1 AJGD patients, these results most likely are repre-

sentative of the mutation(s) in affected Ashkenazi Jewish patients since the mutant enzymes selected had characteristic kinetic and immunologic properties of β-Glc found in a large series of Type 1 AJGD patients (12,13). Also, the kinetic properties of delipidated normal splenic β-Glc and normal or Type 1 AJGD enzymes in crude fibroblast extracts or lymphocytes were essentially identical to those of the respective enzymes reported here (8,13). These results indicated that the respective kinetic properties of the normal and Type 1 AJGD enzymes were consistent in different tissue sources (8,12,13). However, the slightly lower k_{cat} values obtained with the delipidated normal splenic enzyme, compared to the placental enzyme, suggest minor differences in kinetic properties of β-Glc (or its different molecular forms) from various tissues. Based on these considerations, the respective k_{cat} values for the normal β-Glc and the Type 1 AJGD preparations were similar with each of six different substrates (Table 6) representing two different types of leaving groups.

These data provide insight into the nature of the molecular pathology of the deficient enzyme activity in Type 1 AJGD. Previous studies have indicated that the inhibitors, glucosylsphingosine (8,13) and sphingosine (12), have altered affinities for the active site of the Type 1 AJGD enzyme. The present studies indicated a similar decrease (5-fold) in affinity (K_i value) of CBE for the mutant active site. These data suggested that a particular domain or region within or near the mutant active site alters the function of the active site (9,12,13). Immunologic studies with polyclonal (13,16) and monoclonal (27) antibodies have indicated that the normally processed β-Glc protein in Type 1 AJGD tissues was present at about 30 to 100% of nor-

mal levels, but that the enzymatic activity per mg of CRIM was 8- to 20-fold decreased (13,15,16,27). The minor decrease in k_{cat} found in these studies implies that a large percentage of detectable CRIM in previous studies was catalytically inactive enzyme protein. Thus, it appears likely that the defect within or near the Type 1 AJGD active site leads to *in vivo* lability of the enzymatic activity, possibly due to a selectively increased suseptibility to proteases.

Chapter Six

Inhibition Studies Using Glucose Analogues and pH Variation to Characterize the Normal and Gaucher Disease Acid β-Glucosidase Glycon Binding Domains

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This work is in review in the Archives of Biochemistry and Biophysics.

Materials

The following were from commercial sources: Triton X-100, methyl-β-glucoside, 1-thio-β-glucose, 5-thioglucose, streptozotocin [2-deoxy-2-(3'-methyl-3'-nitrosoureido)-D-glucose], δ-gluconolactone, α- and β-glucose-1-phosphate, 6-deoxy-glucose, glucosamine, glucose-6-phosphate and human serum albumin (Sigma Chemical Co., St. Louis, MO), N-methyl-D-glucamine, 1-deoxy-glucose, D-glucal (1,2-deoxy-1,2-dehydro-D-glucose), 3-O-methyl-β-D-glucose, 6-deoxy-6-amino-glucose, (U.S. Biochem, Cleveland, OH) and 6-deoxy-6-amino-allose (ICN-K and K, Plainville, NY), 2-deoxy-2-fluoro-glucose, 3-deoxy-3-amino-glucose, sodium taurocholate (Calbiochem, Los Angeles, CA), castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine; Boehringer-Mannheim, FRG), NBD-dodecanoic acid (NBD-C_{12}; 12-[N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl] aminododecanoic acid) and NBD-hexanoic acid (NBD-C_6, 6-[7-nitrobenz-2-oxa-1,3-diazol-4-yl] aminohexanoic acid) (Molecular Probes, Junction City, OR), and 4-methylumbelliferyl-1-O-β-D-glucoside (4MU-Glc) (RPI, Mount Prospect, IL). 4MU-Glc was recrystallized from ethanol prior to use. All other reagents were used directly.

The following compounds were prepared and purified as described: deoxynojirimycin (dNM; 1,5-dideoxy-1,5-imino-D-glucitol) and nojirimycin (5-deoxy-5-amino-glucopyranose) (16), N-decyl-dNM and N-butyl-dNM (C_{10}-dNM and C_4-dNM; 17), NBD-C_{12}- and NBD-C_6GCs (18), CBE (19), 1-deoxy-1-amino-β-D-glucose (β-glucosylamine; 20), glucosylsphingosine (GS) (21), sphingosine (trans-1,3-diol-2-amino-4-octa-decene) (22), 3-bromopyridinium-N-β-D-glucoside (23) and 1,5-dideoxy-1,5-imino-D-galactitol (Legler, G. and Pohl, S.; unpublished data).

Methods

Enzyme Sources. Normal placental acid β-glucosidase was purified by affinity chromatography on N-alkyl-dNM-Sepharose (24). Homogeneity of the preparations was confirmed by a single protein species on SDS-polyacrylamide gel electrophoresis and a single N-terminal amino acid sequence (24) which was colinear with that predicted from the acid β-glucosidase cDNAs (3,4). These pure preparations were shown to contain one catalytic amino acid per subunit (25). The spleens from 25 and 54 year old Type 1 AJGD patients were obtained at splenectomy and frozen at -20°C until used. The mutant splenic acid β-glucosidases were purified either by hydrophobic chromatography (26) or by N-alkyl-dNM-Sepharose affinity chromatography (24). The final respective preparations were about 500- and 7500-fold enriched and several protein bands were observed on SDS-polyacrylamide gel electrophoresis. These two preparations of differing purity were used as controls for the potential effects of contaminants, since homogeneous mutant enzymes could not be obtained in an active form. The active mutant enzyme in these preparations bound CBE with a 1:1 (mole CBE/mole 56,000 dalton subunit) stoichiometry indicating a single catalytic site per subunit (5). No major differences in the k_{cat} values for several substrates (5), apparent inhibitory constants or $K_m$app values were observed with either Type 1 AJGD splenic preparation. Using immunoblots, the molecular weights of the normal and Type 1 AJGD acid β-glucosidase, after complete deglycosylation with N-glycanase™ (Genzyme, Boston, MA), were identical, M_r=56,000 (data not shown). The immunologic and kinetic

properties of the acid β-glucosidase in the Type 1 AJGD spleen preparations were representative of those from a large series of Type 1 AJGD patients (11,12).

Kinetic Methods. Hydrolysis of NBD-C_{12}-GC, 4-nonylumbelliferyl-β-glucoside, or 4MU-Glc (5,6) was determined fluorometrically. Except where noted, the reaction mixture (0.2 ml) contained phosphate/citrate buffer (ionic strength was constant; μ = 0.07 M), 1 mM β-mercaptoethanol and 1.25 mM EDTA (Buffer A) as well as Triton X-100 (0.8 to 4 mM), sodium taurocholate (4.65 mM), substrate and enzyme effectors. The indicated pH values were determined in the reaction mixtures under the experimental conditions. Most assays were conducted at pH 5.75 and 37°C. For the highly purified Type 1 AJGD enzyme, 0.6% human serum albumin was included in the incubation mixtures to maintain enzyme stability: under these conditions, human serum albumin had no effect on the apparent kinetic constants of the pure normal enzyme or the 500-fold enriched mutant splenic acid β-glucosidase.

The following terminology is used throughout; competitive (K_{is} = finite, $K_{ii} = \infty$), uncompetitive ($K_{is} = \infty$, $K_{ii}app$ = finite), and mixed (K_{is} and K_{ii} = finite) inhibition. All kinetic constants were apparent values ($K_{is}app$, $K_{ii}app$, etc.). Since many of the inhibitors studied had linear intersecting patterns (with $K_{is}app < K_{ii}app$) using 4MU-Glc or NBD-C_{12}-GC as substrates, these compounds also could be designated mixed competitive/noncompetitive to indicate their predominantly competitive nature. For the more potent inhibitors, $K_i app$ values were determined with several substrate concentrations (0.1 to 10 K_m) from Lineweaver-Burk plots and the appropriate slope (for $K_{is}app$) and 1/v-

intercept (for $K_{ii}app$) replots (27) which were evaluated by the least-squares method.

For convenience, the $K_{is}app$ and $K_{ii}app$ values for less potent inhibitors were determined from the equations

$$K_{is}app = \frac{[I]}{\frac{\text{slope (I)}}{\text{slope (0)}} - 1} \qquad K_{ii}app = \frac{[I]}{\frac{\text{intercept (I)}}{\text{intercept (0)}} - 1} \qquad (1)$$

where slope (__) or intercept (__) were from Lineweaver-Burk plots in the presence (I), or absence (0) of the inhibitor using 4MU-Glc or NBD-C_{12}-GC as substrates. $K_{is}app$ and $K_{ii}app$ values of the potent inhibitors were within ±20% by either method and with either substrate for representative inhibitors. The amount of enzyme was adjusted to ensure that less than 5% of substrate was hydrolyzed during the 0.5 to 2 h incubations at 37°C. IC_{50} values (the concentration of inhibitor which resulted in 50% inhibition) were determined with 4MU-Glc (4 mM) as substrate. Within the time frame of these incubations, all inhibitors were rapidly reversible, except CBE which bound covalently (25). The values represent the mean of at least three experiments conducted in duplicate. $V_{max}app$ values were reproducible within ± 7%. All plots and replots were linear with correlation coefficients greater than 0.990. Comparative inhibitor studies with the normal and Type 1 AJGD enzymes were conducted in matched sets with exactly equal incubation times to control for potential instabilities of the inhibitors (e.g., δ-gluconolactone and β-glucosylamine).

Ionization values (pK_e; the acid dissociation constant for an amino acid on the enzyme) for kinetic parameters were determined from

log (V_{max}app, V_{max}app/$K_m$app or K_{is}app) vs pH plots (28). For these experiments, the enzymes were stable at all pH values in the presence of substrate, 4 mM Triton X-100 and 4.65 mM taurocholate. 4MU-Glc was used as substrate since it does not ionize in this pH range. Acid dissociation values (pK_a) for inhibitors, under the experimental conditions, were determined by titration: dNM (6.40 ± 0.10); N-C_{10}- and N-C_4-dNM (6.30 ± 0.10); castanospermine (5.89 ± 0.10), nojirimycin (5.18 ± 0.10) and β-glucosylamine (5.30 ± 0.10).

Effect of Inhibitors on CBE Inactivation Rates. The effects of various inhibitors on the inactivation rate (k_{app}) of acid β-glucosidase by CBE were determined as described to ensure stability of the enzyme in the absence of substrate (5). Briefly, enzyme, inhibitor and CBE (100 μM) were incubated for the indicated time at 22°C in Buffer A, pH 5.5, containing 0.5% each of Triton X-100 and human serum albumin. The mixtures then were diluted 1000-fold with Buffer A (pH 5.75), containing 4 mM Triton X-100 and 4.65 mM taurocholate, and the activity was determined with 4MU-Glc (4 mM) as substrate. The data were plotted according to the equation (29):

$$\ln \frac{E}{E_0} = -k_{app}\, t \qquad (2)$$

where E and E_0 are the remaining enzymatic activity at time t and the initial enzyme activity, respectively. Control studies indicated that the enzyme activity was stable under these conditions for up to 4 h. The K_{is}app and K_{ii}app values for the various reversible inhibitors were determined according to eq 1 in Buffer A (pH 5.5), containing 0.5%

Triton X-100 and 0.5% human serum albumin: i.e., under the CBE inactivation conditions.

Results

Interaction of Acid β-Glucosidase with Glucose Analogues. To define the effect of hydroxyl group configuration or substituent groups on interactions of glycons with the normal and the Type 1 AJGD acid β-glucosidase, the type of inhibition and the respective $K_i$app values were determined for a variety of glucose epimers and derivatives (Table 7). The values represent the results obtained with the 4MU-Glc substrate; within experimental error, the same type of inhibition and K_{is}app and K_{ii}app values were obtained with the more potent inhibitors and the NBD-C_{12}-GC or 4-nonylumbelliferyl-β-glucoside substrates. All glycons were linear intersecting inhibitors with the same type of inhibition for the normal and Type 1 AJGD enzyme sources. In general, the glycons were mixed competitive/noncompetitive inhibitors with K_{ii}app = 2 to 4 K_{is}app except for β-glucose, δ-gluconolactone and 3-deoxy-3-amino-glucose which were competitive and β-glucose-1-phosphate which was uncompetitive. Although no particular hydroxyl group appeared necessary for interaction, the configuration of these groups or the introduction of a charged group did influence the K_{is}app values for the glycons, relative to that for β-glucose. The C-1 anomers of glucose bound equally poorly to either the normal or Type 1 AJGD enzymes, while the C-2, C-3 and C-4 epimers (α- or β-mannose, D-allose or β-galactose, respectively) had increased K_{is}app values. The 1-deoxy, glucal and 1-thio derivatives had similarly decreased K_{is}app values (5- to 9-fold)

TABLE 7

Apparent Inhibition Constants for D-Glucose Derivatives with Normal or Type 1 AJGD Acid β-Glucosidases (37°C, pH 5.75)

Inhibitor	K_{is}(Normal)	K_{is}(AJGD)	$\frac{K_{is}(AJGD)}{K_{is}(Normal)}$
	(mM)	(mM)	
C_1 Derivatives:			
β-D-glucose	220 ± 50	300 ± 64	1.4
α-D-glucose	300	350	1.2
1-O-Me-β-glucose	550	500	0.9
1-O-Me-α-glucose	550	84 ± 17	0.15
1-deoxy-D-glucose	35 ± 7	50 ± 10	1.4
Glucal	24 ± 6	34 ± 10	1.4
1-thio-β-D-glucose	50 ± 11	50 ± 14	1
β-glucosylamine	0.35 ± 0.06	6.05 ± 0.91	17.3
N-methyl-glucamine	3.0 ± 0.7	15 ± 31	5
δ-gluconolactone	0.30 ± 0.07[a]	1.80 ± 0.45	6
α-glucose-1-PO_4	50 ± 10	> 200	>4
β-glucose-1-PO_4	0.53 ± 0.11[b]	0.55 ± 0.13	1[c]
N-Br-pyridinium-β-glucose	6.4 ± 1.5	13.5 ± 4.0	2.1
C_2 Derivatives:			
β-mannose	> 500	> 500	--
α-mannose	> 500	> 500	--
2-deoxy-glucose	250 ± 62	250 ± 60	1
D-glucosamine	∿ 250	63 ± 13	≅0.4
N-acetylglucosamine	> 500	100 ± 20	--
2-fluoro-2-deoxy-glucose	75 ± 15	75 ± 18	1
Streptozotocin	42 ± 10	80 ± 17	1.9
C_3 Derivatives:			
D-allose	> 250	> 250	--
3-deoxy-3-amino-D-glucose	1.90 ± 0.48	8.4 ± 2.5	4.4
3-O-Me-D-glucose	∿ 250	> 250	>1
C_4 Derivatives:			
β-galactose	> 500	> 500	--
1-O-Me-β-galactose	> 500	> 500	--

TABLE 7 - Continued

C_5 Derivatives:			
5-deoxy-5-thio-D-glucose	> 250	> 250	--
1-deoxynojirimycin	0.053 ± 0.008 0.009[d]	0.56 ± 0.09	10.5
N-C_4-1-deoxynojirimycin	190	470	2.5
Nojirimycin	0.005 ± 0.001 0.0034[d]	0.006 ± 0.001	1.2
Castanospermine	0.007 ± 0.003 0.0029[d]	0.011 ± 0.005	1.6
C_6 Derivatives:			
6-deoxy-glucose	200 ± 48	200 ± 43	1
6-deoxy-6-amino-glucose	∿ 250	∿ 250	--
6-deoxy-6-amino-allose	∿ 300	∿ 300	--
Glucose-6-PO_4	> 500	> 500	--
Glucosamine-6-PO_4	250		

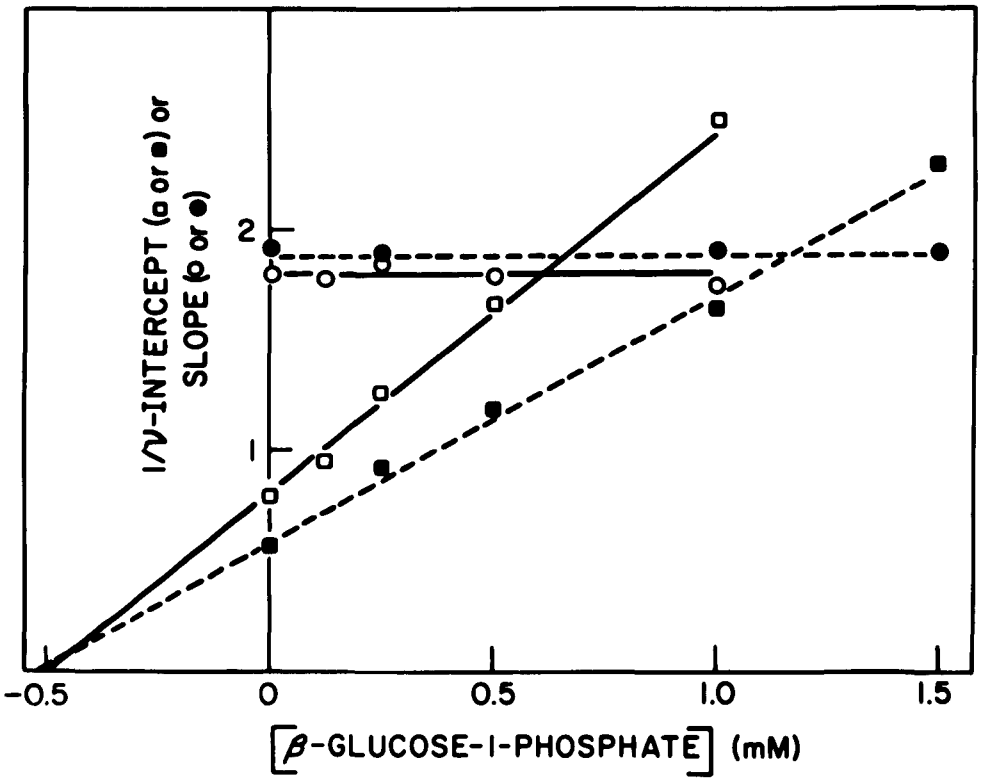
[a] K_{is} value is an approximate value since δ-gluconolactone is unstable ($t_{1/2}$ ≅20 min) at pH 5.75 and the hydrolytic product gluconic acid has K_{is} ≅ 60 mM.
[b] $K_i$app value for uncompetitive inhibition.
[c] Ratio of uncompetitive values.
[d] pH-independent K_{is}app value according to eq 4.

for either enzyme. The introduction of a β-amino group at C-1, i.e., β-glucosylamine, had two effects: 1) the K_{is}app values for either enzyme were decreased by 20-to 600-fold and 2) the K_{is}app(Type 1 AJGD)/ K_{is}app(normal) increased from near equality to 17; a ratio of six was obtained with δ-gluconolactone. The C-2 derivatives with acidifying groups (fluoro or nitroso) had equally decreased K_{is}app values with either enzyme. D-Glucosamine or its N-acetyl derivative were poor inhibitors but were 3- to 5-fold more potent against the mutant enzymes. Of the C-2, C-3 or C-4 glucose derivatives, 3-deoxy-3-amino-glucose was the most potent inhibitor of either enzyme. The 6-phosphate derivative did not inhibit either enzyme, while 6-deoxy-6-amino-glucose or its C-3 epimer had K_{is}app values similar to those for glucose.

In comparison, the substituent at C-5 of the glucopyranose ring had pronounced effects on the K_{is}app values. The 5-deoxy-5-thio derivative bound poorly to either enzyme whereas the 5-deoxy-5-imino derivatives had K_{is}app values that were 4×10^3 (dNM) to 4×10^4 (castanospermine and nojirimycin) less than those for glucose. At pH 5.75, K_{is}app(Type 1 AJGD)/K_{is}app(normal) was about ten with dNM but nearly one with nojirimycin and castanospermine. The IC_{50} value for 1,5-dideoxy-1,5-imino-D-galactitol (galacto-dNM) was greater than 20 mM with the normal enzyme.

β-Glucose-1-phosphate was unique among the inhibitors, since uncompetitive inhibition was observed with either the normal or mutant enzymes using 4MU-Glc as substrate (Fig. 18). β-Glucose-1-phosphate ($\leq$ 10 mM) did not inhibit NBD-C_{12}-GC hydrolysis by either enzyme.

Figure 18



Inhibition of normal (● or ■) or Type 1 AJGD acid β-glucosidase (○ or □) hydrolysis of 4MU-Glc by β-glucose-1-phosphate. Slope (○) or 1/v-intercept (□) from double reciprocal plots were multiplied by 10^5 or 10^3 for the normal or Type 1 AJGD enzymes, respectively.

Effect of pH on Kinetic Parameters. Using 4MU-Glc as substrate, the variation of V_{max}app or V_{max}app/$K_m$app for the normal enzyme with pH (pH = 4.20-7.25) described bell-shaped curves which fit a diprotic model (eq 3) with pK_1 = 4.7 ± 0.2 and pK_2 = 6.7 ± 0.2 with an optimum at pH 5.75: V_{max}app/$K_m$app and $K_m$app varied by an order of magnitude and about 2-fold, respectively, at 4.2 $\leq$ pH $\leq$ 7.25 (data not shown). Essentially identical results for V_{max}app or V_{max}app/$K_m$app vs pH were obtained with the AJGD Type 1 enzyme where pK_1 = 4.7 and pK_2 $\simeq$ 6.9 with an optimum at pH = 5.8 (data not shown).

$$EH_2 \underset{}{\overset{K_1}{\rightleftharpoons}} EH^- \overset{K_2}{\rightleftharpoons} E^{2-} \qquad (3)$$

These results indicated that proper protonation of two groups on the normal or Type 1 AJGD enzymes was required for catalysis of 4MU-Glc and the lack of plateaus at the pH extremes of the V_{max}app or V_{max}app/$K_m$app vs pH curves indicated that a monoprotonated enzyme, EH^-, is the probable active enzyme species whereas EH_2 and E^{2-} were inactive (29).

With the normal enzyme, the variation of K_{is}app(plotted as $1/K_{is}$app) values with pH for dNM, N-C_{10}-dNM, castanospermine and nojirimycin are shown in Figures 19A-D, respectively. For each inhibitor, the data were fitted to several models (30) but, the best fit was to eq 4 for the unprotonated inhibitor binding to EH_2 and EH^-. Eq 4 was derived under rapid equilibrium assumptions with $K_1^{EH^-} = K_1^{EH^-I}$. It should be noted that eq 4 cannot determine the number of mutually exclusive pK_2 = 6.7 residues. The presence of two or more mutually exclusive groups will not alter the shape of the curves but will alter its height due to additional constant terms in the denominator which

Figure 19

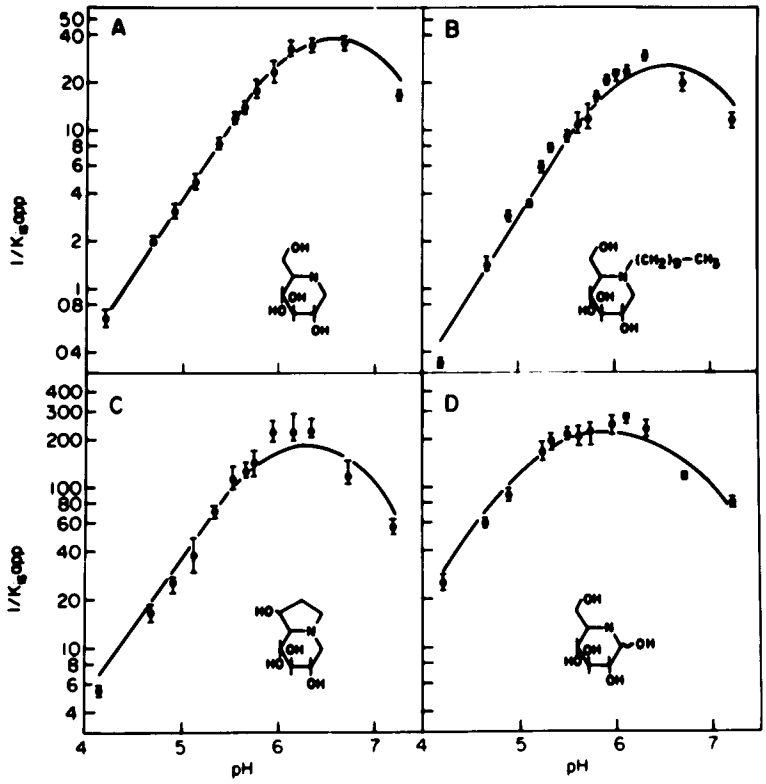


Figure 19:

Effect of pH on $1/K_{is}app$ values of dNM (A), N-C_{10}-dNM (B), castanospermine (C) or nojirimycin (D) for normal acid β-glucosidase hydrolysis of 4MU-Glc. The symbols represent the mean values and ranges for at least six determinations. The curves represent the best fit to eq 4 assuming that the unprotonated form of the inhibitors bound to the EH^- and EH_2 forms of the enzyme. $1/K_{is}app$ values are $(mM)^{-1}$ in A, C and D, and $(\mu M)^{-1}$ in B determined at 37°C.

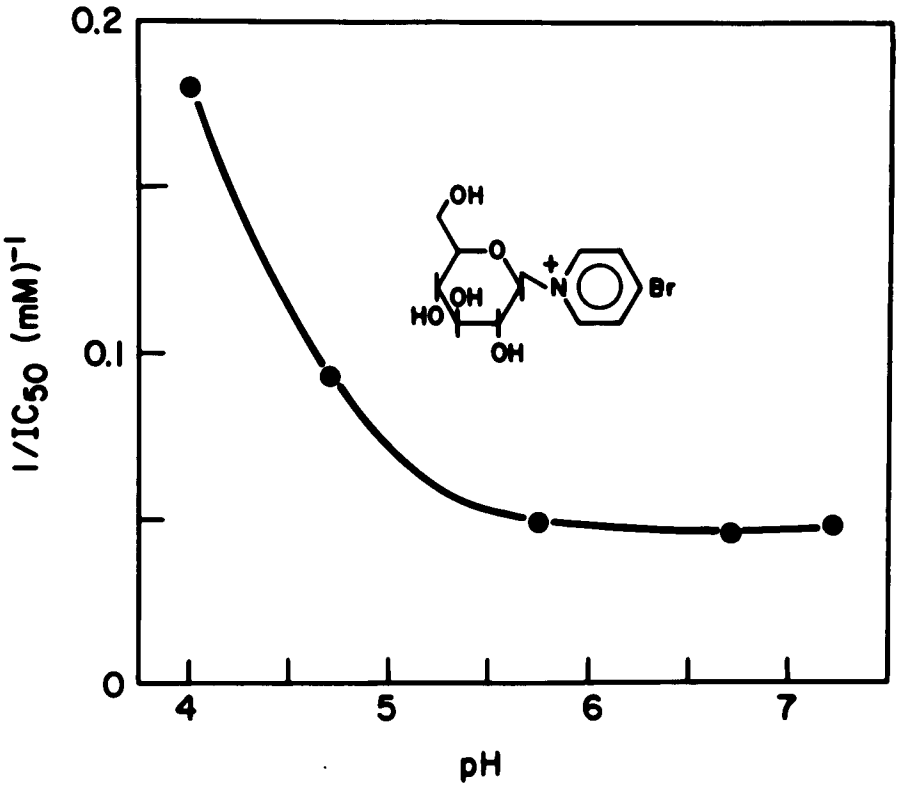
relate the K_i values for each $pK_2 = 6.7$ term.

$$K_{is}app = K_{is}\left(1+\frac{K_2}{[H^+]}+\frac{[H^+]}{K_a}+\frac{K_2}{K_a}\right) \qquad (4)$$

For the glycon inhibitors, the pH-independent $K_{is}app$ values (K_{is} in eq 4) are shown in Table 7; the corresponding value for C_{10}-dNM was 13 nM. Because of the instability of β-glucosylamine at pH < 5.0, the $K_{is}app$ values had large variations at low pH; only models for the unprotonated form of this inhibitor binding to the protonated enzyme ($pK_2 = 6.7$) fit reasonably well (pH-independent $K_{is}app \geq 0.22$ mM, data not shown). The $1/IC_{50}$ vs. pH profile for the permanently cationic, competitive inhibitor, 3-bromopyridinium-N-β-D-glucoside (Fig. 20), indicated that it had greater affinity for EH_2 and EH^- than E^{2-} but apparently bound to all enzyme forms and that residues not essential to the catalysis of 4MU-Glc were involved.

Effect of Taurocholate on $K_{is}app$ of Inhibitors. Figure 21 shows the effect of increasing taurocholate concentration (in the presence of 4 mM Triton X-100) on the $K_{is}app$ of β-glucosylamine, dNM, C_{10}-dNM and castanospermine. To facilitate comparison of these inhibitors of markedly differing potency, the data are plotted as the percent of the $K_{is}app$ value obtained in the absence of taurocholate. The $K_{is}app$ values for β-glucosylamine, dNM, C_{10}-dNM and castanospermine at 19 mM taurocholate were 0.31 mM, 0.031 mM, 21 nM and 7 μM, respectively. In contrast to the marked effects on the $K_{is}app$ values for the other inhibitors, the $K_{is}app$ values for castanospermine was unaffected by increasing taurocholate concentration. Enhanced inhibitory potency of

Figure 20



Effect of pH on $1/IC_{50}$ values, $(mM)^{-1}$,
for 3-bromopyridinium-N-β-D-glucoside, a permanently cationic,
competitive inhibitor of the normal enzyme.

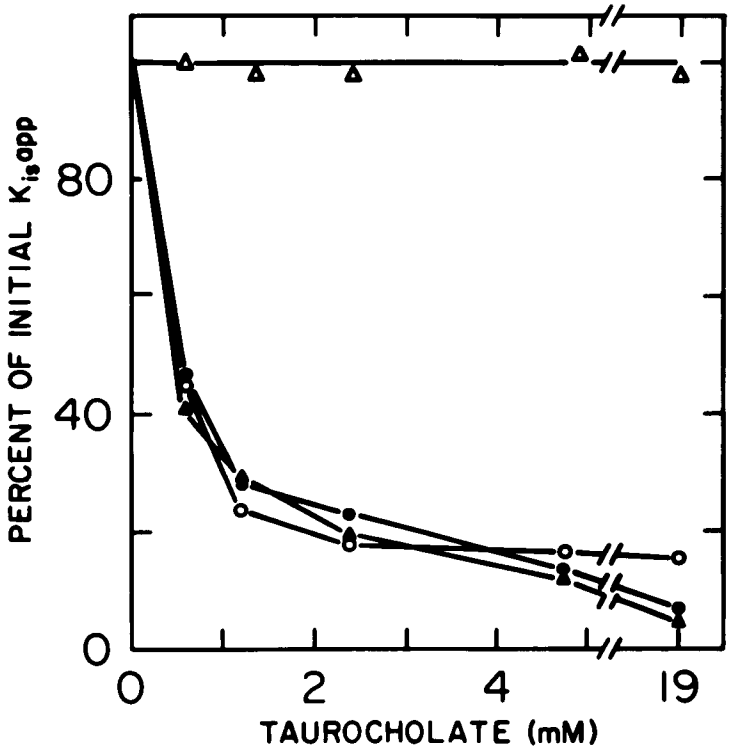
δ-gluconolactone by increasing amounts of taurocholate has been reported (6).

Effect of Inhibitors on CBE Inactivation Rates. Due to the mixed ($K_{is}app < K_{ii}app$) inhibition patterns obtained with most of the inhibitors, the interaction with the active site was inferred by their structural similarity to β-glucose. To obtain supporting evidence for binding of these inhibitors at the active site, the nature of the interactions of representative inhibitors with CBE, a covalent catalytic site-directed inhibitor, was determined. These experiments were conducted with the purified placental enzyme in the presence of 100 μM CBE, 0.5% Triton X-100 and 0.5% human serum albumin. The results were evaluated by three models (eq 5) depending on the competitive ($K_{ii}app = \infty$), mixed ($K_{is}app$ and $K_{ii}app$ = finite) or uncompetitive inhibition ($K_{is}app = \infty$) observed with the respective inhibitors. The respective $K_i app$ values in the absence of taurocholate were obtained as described in Fig. 21 and are shown in Table 8.

$$k_{app} = \frac{k_{max}\,[CBE]}{K_i\,(1 + \frac{[I_a]}{K_{is}}) + [CBE]\,(1 + \frac{[I_a]}{K_{ii}})} \qquad (5)$$

In eq 5, $[I_a]$ is the concentration of the reversible inhibitor and the K_i and k_{max} values for CBE were the apparent dissociation constant ($K_i app$ = 166 μM) and the maximal rate of inactivation (k_{max} = 0.052/min) under these assay conditions (5). Table 8 compares the calculated values for k_{app} (k_{app}^{calc}) from the models (eq 5) with the observed values (k_{app}^{obs}). For 3-deoxy-3-amino-glucose, k_{app}^{obs} was

Figure 21



Effect of increasing taurocholate concentration on the K_{is}app of β-glucosylamine (O), dNM (●), N-decyl-dNM (▲) and castanospermine (Δ). The results are plotted as the percent of the respective K_{is}app values obtained in the presence of 4 mM Triton X-100 alone.

TABLE 8

Interaction of CBE and Glycon Inhibitors (22°C, pH 5.50)

Inhibitor (mM)[a,b]	k_{app}^{calc}	k_{app}^{obs}
	(min^{-1})	(min^{-1})
no added glycon	0.0195	0.021 ± 0.003
3-deoxy-3-amino-glucose (13)	0.0087[c]	0.0087
K_{is} = 6.53 mM	0.0065[d]	
	0.0111[e]	
dNM (0.25)	0.0087[c]	0.0083
K_{is} = 0.125 mM	0.0084[d]	
K_{ii} = 1.14 mM	0.0112[e]	
N-C_{12}-dNM (0.0002)	0.0086[c]	0.0087
K_{is} = 0.2 μM	0.0083[d]	
K_{ii} = 1.5 μM	0.0111[e]	
GS (0.0025)	0.0155[c]	0.0138
K_i = 6 μM	0.0138[d]	
	0.0169[e]	
β-glucose-1-phosphate (6.4)	0.0063[c]	0.0088
K_i = 3.83 mM	0.0045[d]	
	0.0087[e]	

a Reactions conducted in the presence of 0.5% each of Triton X-100 and human serum albumin,

b Concentration of inhibitor used in the presence of 100 μM CBE,

c,d,e Competitive, mixed or uncompetitive models, respectively (see eq 5 in text).

nearly identical to that calculated from the competitive model. With dNM or N-C_{12}-dNM, the competitive ($K_{ii}app = \infty$) or mixed ($K_{ii}app \geq 9$ $K_{is}app$; Table 8) models conformed closely to k_{app}^{obs}. With GS only k_{app}^{calc} for the *noncompetitive* model ($K_{is}app = K_{ii}app = 6$ μM) corresponded to k_{app}^{obs}. For β-glucose-1-phosphate, only the uncompetitive model conformed to the data.

Discussion

In this communication, kinetic studies are reported which provide insight into the properties of the glycon binding domain within the active site of human acid β-glucosidase. These studies also indicate that the mutation(s) in the acid β-glucosidase structural gene which results in Type 1 AJGD leads to an altered acid β-glucosidase active site function. Previous studies of human acid β-glucosidase, which focused on the effects and interactions of lipoidal modifiers have suggested the presence of at least three active site domains, a hydrophilic catalytic site and two hydrophobic domains, the aglycon binding site and a "third" domain (6). The properties of the latter two domains will be reported separately (31). The catalytic site was proposed to recognize the hydrophilic glycon head groups of inhibitors and substrates as well as CBE. Since Asp^{443} has been identified as critical to catalytic activity (25), i.e., the catalytic amino acid for the proposed nucleophilic attack of the reactive intermediate (23), a more informative term, the glycon binding domain, will be used for the recognition region for glycon head groups: Asp^{443} residue is in or near

this domain. The present studies indicate that an amino acid with pK_e = 6.7 is important for binding of inhibitors or hydrolysis of substrates and is in proximity to the glycon binding domain (Fig. 22). The interactions of particular glycon inhibitors with this domain was specifically affected by the Type 1 AJGD mutation(s).

Specificity of glycon binding could be assessed from the inhibition data for monosaccharides only to a limited extent, since sugars epimeric at C-2, C-3 or C-4 had K_{is}app values outside the accessible concentration range, i.e., $\geq$ 2.5-fold larger than that for glucose. Compared to the glucosyl analogues, β-galactosyl sphingosine and 1,5-dideoxy-1,5-imino-D-galactitol were poor inhibitors [IC_{50} > 2 mM (6) and 20 mM, respectively] indicating the importance of the configuration at the C-4 hydroxyl. D-Glucal or 1-deoxy-glucose were about 7- to 10-fold more potent and 3-methoxy-glucose and 2-deoxy-glucose about as potent inhibitors as glucose, whereas 3-deoxy-3-amino-glucose was about 100-fold more potent than glucose. These results indicated that the binding of glucose to the glycon binding domain requires hydrogen bonding to the C-3 and C-4 hydroxyl groups in a preferred configuration, but those at C-1 and C-2 were less important. In comparison, the almond β-glucosidase was not very specific for the configuration at C-4, i.e., p-nitrophenyl- β-glucoside and -β-galactoside were hydrolyzed with comparable rates and glucosylamine and galactosylamine had almost identical $K_i$app values (32).

Large effects on glycon interactions were observed by the introduction of substituents on the glucose ring. At C-1, α-phosphate (a negative charge) or β-thiol groups increased the affinity for the normal enzyme by 5- to 7-fold. In comparison, a β-anomeric negative (phos-

phate) charge at C-1 resulted in about a 500- to 700-fold increased affinity for the enzyme. However, the inhibition of 4MU-Glc activity by β-glucose-1-phosphate was uncompetitive. Although true uncompetitive inhibition is rare in unireactant systems, the fact that $k_{app}^{obs} = k_{app}^{calc}$ for β-glucose-1-phosphate interaction with CBE fit only the uncompetitive model (Table 8) indicated a stereospecific interaction of this reversible inhibitor with a positively charged group on the enzyme after the 4MU-Glc substrate was bound. The lack of inhibition of NBD-C_{12}-GC hydrolysis by β-glucose-1-phosphate indicates that the presence of the natural substrate in the active site was sufficient to exclude this compound from the enzyme or that the rate-determining step for GC and 4MU-Glc hydrolysis differed.

The nature of groups on the enzyme for interaction with C-2 of glucose was unclear. A positively-charged group on the normal enzyme appeared to be in proximity to the C-2 of glucose, since the acidifying fluoro and nitroso (streptozotocin) derivatives had 5- to 7-fold decreased K_{is}app values. However, the presence of such a positively-charged group on the enzyme should have resulted in a K_{is}app (glucosamine) > 500 mM (not K_{is}app $\simeq$ 250 mM; Table 7), since the repulsive effect on the primary amine group would be much larger than the expected ion-dipole interactions with the 2-fluoro and 2-nitroso groups. These results indicate a laxity of fit for these poor inhibitors at the glycon binding domain. The results with glucosamine contrast with its potent inhibition of the almond β-glucosidase (33).

The bell-shaped curve for the pH-dependency of V_{max}app or observed velocity has been obtained with numerous other enzymes or crude and purified acid β-glucosidase, respectively. In the present studies,

V_{max}app or V_{max}app/ $K_m$app varied by over an order of magnitude whereas $K_m$app for 4MU-Glc was nearly constant (about 2-fold variation) from $4.20 \leq pH \leq 7.25$: i.e., the variation in V_{max}app/$K_m$app with pH was due entirely to V_{max}app. Importantly, this is the expected result, if $K_m$app = K_s, the true dissociation constant under these experimental conditions (34). The identification of two ionizable groups on acid β-glucosidase (pK_1 = 4.7 and pK_2 = 6.7) with an optimum of pH = 5.75 was explained most easily by eq 3 with EH^- as the active and EH_2 and E^{-2} the inactive enzyme species. Consistent with these findings was the identification of Asp^{443} as the residue affinity labelled by Br-CBE, an active site directed covalent inhibitor of this enzyme (25). Since Asp^{443} must be the nucleophile in the formation of the ester linkage of Br-CBE to the enzyme and most likely the nucleophile in catalysis, pK_1 = 4.7 can be assigned to this residue. Consequently, the pK_2 = 6.7 residue is the H^+ donor for the proton transfer in the hydrolytic mechanism (see below). The finding that the active site of a similar enzyme, bovine acid β-glucosidase, was strongly shielded from the aqueous solvent (17), suggests a shift in particular pK_e values for active site residues and that the pK_2 = 6.7 residue in the active site of the human enzyme could correspond to a carboxylic acid as has been described for lysozyme (35).

The variation with pH of the K_{is}app for the 5-amino-5-deoxy-glucosides indicated the requirement of at least one proton in the EI complex. The finding of only pK_a (inhibitor ionization) and pK_2 = 6.7 (enzyme ionization) dependency of K_{is}app with these compounds indicated that the binary complex was EH^-I and EH_2I, i.e., the unprotonated inhibitor bound equally well to either protonated enzyme form. This

conclusion required the studies with nojirimycin and castanospermine, since the pK_a (= 6.3) of dNM was sufficiently close to pK_2 to result in an ambiguity of the true inhibitory forms as was the case with almond β-glucosidase (33). Although the modeling studies conformed extremely well to the unprotonated inhibitor binding to the glycon binding domain, a model for protonation of the inhibitor within this domain is kinetically equivalent provided that 4MU-Glc is not a "sticky" substrate: i.e., this substrate is hydrolyzed at a rate that is much slower than its dissociation rate from the enzyme (30). The finding that the K_m for this substrate is a thermodynamic constant indicates that it has little, if any, "stickiness". The results with N-C_{10}-dNM exactly paralleled those with dNM, indicating that no additional ionizable groups within the active site were required for interaction with the alkyl group, i.e., the increased potency of N-C_{10}-dNM (10^3-fold) was the result of hydrophobic interactions.

To account for the increased inhibitory potency of particular substrates or product analogues, investigators have used transition state theory. This theory predicts favorable interactions of substrates and the enzymes leading to greatly increased affinity during the transition state (36). Consequently, very potent reversible inhibitors ($K_{is}/K_m = 10^{-4}$ to 10^{-8}) of an enzymatic reaction are candidate transition state analogues from which mechanistic deductions can be made. However, a true transition state analogue should not only be a potent inhibitor, but should bind only to the active enzyme species (37). With the human acid β-glucosidase, the unprotonated 5-amino-5-deoxyglucoside inhibitors did not have a $pK_1 = 4.7$ dependency and bound equally well to the EH^- (active) and EH_2 (inactive) enzyme species.

Therefore, they cannot be true transition state analogues and their inhibitory potencies arise from favorable hydrogen bonding and/or steric effects.

The finding that $K_{is}app(N\text{-}C_4\text{-}dNM)/K_{is}app(dNM) \geq 4$ and $K_{is}app(castanospermine)/K_{is}app(dNM) \geq 0.32$, using pH-independent $K_{is}app$ values, indicated a deterimental steric effect of the C_4 chain compared to the rigid planar ethyl bridge of castanospermine. As illustrated in Fig. 22, this could be explained by the N group of dNM interacting primarily with a cationic residue ("Y") on the enzyme with $pK_a = 6.7$ but dNM could "wobble" from the more favorable fit with "Y". In comparison, the planar ethyl bridge of castanospermine or the C-1 hydroxyl group of nojirimycin could constrain the N group into more favorable interactions with "Y" leading to increased inhibitory potency. This is supported by the lack of effect of taurocholate on $K_{is}app$ (castanospermine) which indicated that this compound had optimum interaction with the enzyme under a variety of assay conditions whereas dNM and β-glucosylamine did not. Because of its $pK_2 = 6.7$ dependency, the C-1 amino group of β-glucosylamine also would interact with the cationic "Y", but steric interference near C-1 was evidenced by the 10-fold increased $K_{is}app$ of N-methyl-glucamine. These results indicate that the glycon binding domain has important steric or configurational requirements for binding to the ring or C-1 oxygens of glucose.

LeLegerie et al. (29) have proposed two different groups of β-glycosidases based on their relative interactions with cationic (group 1) or basic (group 2) glycosyl derivatives. Based on inhibitor studies and α-deuterium kinetic isotope effects, the hydrolytic mechanism of the group 1 enzymes [e.g., Aspergillus wentii β-glucosidase A_3

Figure 22

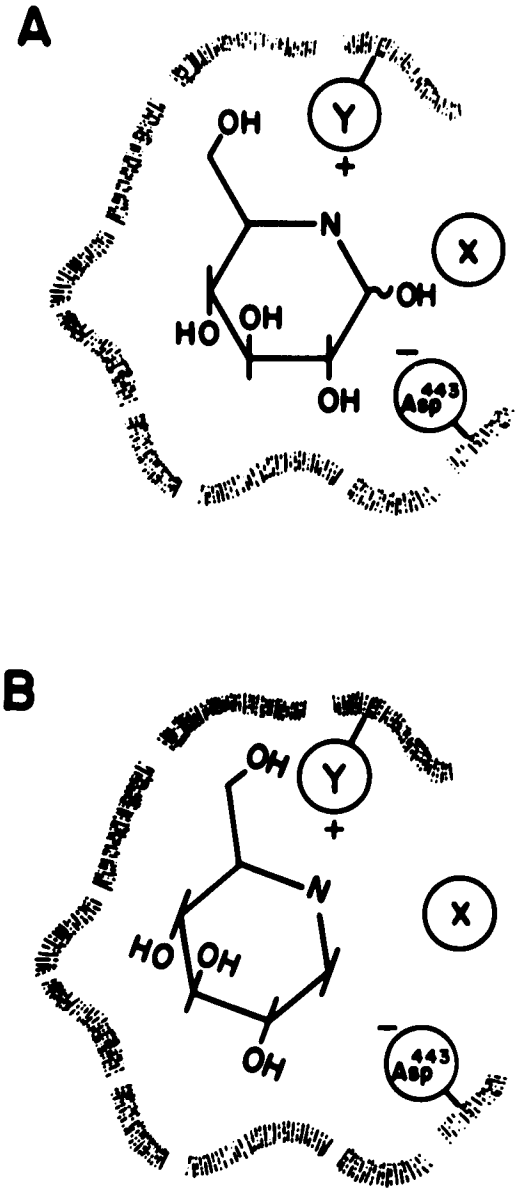


Figure 22:

A schematic representation of the normal or Type 1 AJGD acid β-glucosidase glycon binding domain with bound, unprotonated nojirimycin (A) or dNM (B). The outline of the glycon binding domain is discontinuous since no particular alignment of the amino acid sequence is implied. Asp^{443} is the catalytically necessary amino acid which participates in nucleophilic attack of the O-glucosidic bond. "Y" is the $pK_2 = 6.7$ amino acid for interaction with the ring oxygen of glucopyranose or the unprotonated N of nojirimycin. The group "X" represents an amino acid in the normal active site which interacts with the substituent on C-1 or C-5 of glucose. Other residues for interaction with C-2, C-3, C-4 or C-6 hydroxyl groups of glucose are not specified (see text). The labeled residues can have any orientation above, below or in the plane of the page. "X" is hypothesized to be altered either by direct substitution of a neutral or partially charged amino acid or indirectly by a substitution in a nearby amino acid which alters the position of X in the Type 1 AJGD active site resulting in selectively decreased binding of the unprotonated forms of dNM and β-glucosylamine. Of the designated amino acid residues in the glycon binding domain, only "Y" and Asp^{443} participate in catalysis.

(23)] includes an oxocarbonium-ion reactive intermediate (transition state) which is stabilized by electrostatic interactions. In comparison, the group 2 enzymes [e.g., almond β-glucosidases (33,38)], have been proposed to cleave the O-glycosidic bond by a nucleophilic (S_N2) mechanism or a mechanism with little change in hybridization at the anomeric carbon up to the first irreversible step (39). Since O-glucosidic bond cleavage was nearly complete during the transition state (39) a (largely) covalent glucosyl-enzyme complex might be formed during substrate hydrolysis as suggested for β-galactosidase from E. coli (40,41). This hydrolytic mechanism implies a partial negative-charge accumulation on the oxygen of the glycosidic bond which, presumably, is stabilized by electrostatic interactions with a group in the solvent shielded active site. The findings that the basic forms of 1-amino and 5-imino substituted glucosides are the true inhibitors and the weak affinity of the cationic 3-bromopyridinium-N-β-glucoside indicates that the human acid β-glucosidase belongs to group 2. Greater understanding of the details of the human acid β-glucosidase catalytic mechanism will be required for a fuller appreciation of the transition state during substrate hydrolysis by this enzyme. However, the linearity of the slope and intercept replots with these glycon inhibitors indicate an ordered uni-bi kinetic mechanism for substrate hydrolysis. The kinetic similarities of human acid β-glucosidase to those of almond β-glucosidase suggest that the hydrolytic mechanism includes a proton transfer and nucleophilic attack resulting in an ion pair stabilized complex prior to the release of the first product.

The studies with the Type 1 AJGD splenic enzymes indicated that the catalytic residues were not altered and that the presumed single

amino acid alteration in this protein did not result in large conformational changes at the active site. This conclusion is supported by the following: 1) the normal K_1 and K_2 for V_{max}app and V_{max}app/$K_m$app vs. profile, 2) k_{cat} values for several substrates which were similar to those for the normal splenic acid β-glucosidase, and 3) normal k_{max} values with CBE (5). The finding of normal K_{is}app values (at pH = 5.75) for castanospermine and nojirimycin with the Type 1 AJGD enzymes indicated a highly specific alteration of the glycon binding domain limited to particular inhibitors: i.e., the 10- or 17-fold decreased inhibitory potency (pH = 5.75) of dNM or β-glucosylamine, respectively. This concept is also supported by the nearly normal K_{is}app values for several C-1 and C-2 glucose derivatives (e.g., 1-deoxyglucose, glucal, 2-fluoro-2-deoxyglucose) (Table 7) with the Type 1 AJGD splenic enzymes. The nearly normal $K_m$app values for several different glucosylceramide and synthetic β-glucoside substrates (5) also indicate the proper alignment of residues in the active site for substrate binding and hydrolysis. Based on these data, we are led to the conclusion that the amino acid substitution in Type 1 AJGD has a specific effect at or near the glycon binding domain and involves the residues for interaction with the ring or C-1 oxygens of glucose.

Chapter Seven

The Use of Inhibitors, Alternate Substrates and Amphiphiles to Investigate the Properties of the Normal and Gaucher Disease Acid β-Glucosidase Active Sites

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Materials and Methods

Materials

The following were from commercial sources: Triton X-100, phosphatidyl serine, 2-N-stearyl-dihydrosphingosyl-1-O-β-D-glucoside (Lot No. 1066-0335), and human serum albumin (Sigma Chemical Co., St. Louis, MO), sodium taurocholate and alkyl (C_6- to C_{12}-)-1-O-β-D-glucosides (Calbiochem, Los Angeles, CA), sodium dodecyl sulfate (SDS, British Drug House, Poole, UK), alkyl (C_6-to C_{14}-) amines and alkyl (C_2- to C_{24}-) carboxylic acids (Aldrich Chem. Co., Milwaukee, WI), alkyl [^{14}C]carboxylic acids (C_1 to C_{18} chain lengths) labeled on C_1 (50 to 60 mCi/μmol; C_4 was 13.4 mCi/μmole) (New England Nuclear, Cambridge, MA), NBD-dodecanoic (NBD-C_{12}, 12-[N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl] aminododecanoic) and NBD-hexanoic (NBD-C_6, 6-[7-nitrobenz-2-oxa-1,3-diazol-4-yl] aminohexanoic) acids (Molecular Probes, Junction City, OR), 2-N-lignoceryl-dihydrosphingosyl-1-O-β-D-glucoside (Lot No. 91-063-3) (Miles Labs, Naperville, IL), para-, ortho- and meta- nitrophenyl-β-D-glucosides, 1-and 2-naphthyl-β-D-glucosides and 4-methylumbelliferyl-1-O-β-D-glucoside (4MU-Glc) (RPI, Mount Prospect, IL). 4MU-Glc was recrystalized from ethanol prior to use. All other reagents were used directly.

The following were prepared and purified as described: dNM (1,5-dideoxy-1,5-imino-D-glucitol) [20], N-C_n (C_1, C_4, C_{10}, C_{12} and C_{14})-dNMs [21], and NBD-C_{12}- and NBD-C_6-GCs [22], glucosylsphingosine (GS) [23], and sphingosine (trans-1,3-diol-2-amino-4-octadecene) [24]. The unlabeled and radiolabeled C_1-to C_{24}-GCs (C_n-GCs) were synthesized from GS and the appropriate fatty acids [25] and purified by TLC [22]. Purity of the C_n-GCs was demonstrated by HPLC [26].

Methods

Enzyme Sources. Normal placental acid β-glucosidase was purified by affinity chromatography on N-alkyl-dNM-Sepharose [27]. Homogeneity of the preparations was confirmed by a single protein species on SDS-polyacrylamide gel electrophoresis and a single N-terminal amino acid sequence [27] which was colinear with that predicted from the acid β-glucosidase cDNAs [4,5]. These pure preparations were shown to contain one catalytic amino acid per subunit [6]. The spleens from 25 and 54 year old Type 1 AJGD patients were obtained at splenectomy and frozen at -20°C until used. The mutant splenic acid β-glucosidases were purified either by hydrophobic chromatography [28] or by N-alkyl-dNM-Sepharose affinity chromatography [27]. The final respective preparations were about 500- and 7500-fold enriched and several protein bands were observed on SDS-polyacrylamide gel electrophoresis. These two preparations of differing purity were used as controls for the potential effects of contaminants, since homogeneous mutant enzymes could not be obtained in an active form. The active mutant enzyme in these preparations bound CBE with a 1:1 (mole CBE/mole 56,000 dalton subunit) stoichiometry indicating a single catalytic site per subunit [13]. The presence of acid β-glucosidase II [29] could not be detected in these preparations by immunoprecipitation with monospecific polyclonal antibodies [12,13]. No major differences in the k_{cat} values for several substrates [13], apparent inhibitory constants or $K_m$app values were observed with either Type 1 AJGD splenic preparation. Using immunoblots, the molecular weights of the normal and Type 1 AJGD acid β-glucosidase, after complete deglycosylation with N-glycanase™ (Gen-

zyme, Boston, MA), were identical, M_r=56,000 (data not shown). The immunologic and kinetic properties of the acid β-glucosidase in the Type 1 AJGD spleen preparations were representative of those from a large series of Type 1 AJGD patients [11,12].

Kinetic Methods. Hydrolysis of NBD-C_{12}-GC or 4MU-Glc [7,13] was determined fluorometrically. Except where noted, the reaction mixture (0.2 ml) contained phosphate/citrate buffer (ionic strength was constant; μ = 0.07 M), 1 mM β-mercaptoethanol and 1.25 mM EDTA (Buffer A) as well as Triton X-100 (0.8 to 4 mM), sodium taurocholate (4.65 mM), substrate and enzyme effectors. The indicated pH values were determined in the reaction mixtures under the experimental conditions. The lipoidal compounds in chloroform/methanol (2:1; v/v) were added to dry tubes and the solvents evaporated with nitrogen and, then, under high vacuum for 2 to 4 h. For the highly purified Type 1 AJGD enzyme, 0.6% human serum albumin was included in the incubation mixtures to maintain enzyme stability: under these conditions, human serum albumin had no effect on the apparent kinetic constants of the pure normal enzyme or the 500-fold enriched mutant splenic acid β-glucosidase. Unless otherwise noted, the pH of the reaction mixtures was 5.75 at 37°C. The amount of enzyme was adjusted to ensure that less than 5% of the substrate was hydrolyzed. Reactions were terminated after 0.5 to 2 h at 37°C.

For the radioactive C_n-GCs, hydrolyses were conducted as above and stopped with 0.5 ml of chloroform/methanol (2:1, v/v). After vigorous agitation of the mixtures and centrifugation (2000 x g; 10 min), the aqueous layers were removed, the solvents in the organic layers

were evaporated under nitrogen and the residues were resolubilized in 20 to 50 µl of chloroform/methanol (2:1, v/v). The ceramides were quantitatively partitioned to the organic phase but the variable amounts of the respective C_n-GCs contaminated this phase. Therefore, ceramides were resolved from the GCs by TLC (Silica G, Analtech, Newark, DE) using the solvent system, chloroform/methanol/water (85:15:1.5, v/v/v). The particular ceramide was located by the R_f value of the respective ceramide standards, the silica was scraped into a scintillation vial, heated in 1 ml of chloroform/methanol (2:1, v/v) at 37°C for 0.5 h, and, then, the radioactivity was determined using OCS (Amersham, Arlington Heights, IL) as the scintillant. Blank values (i.e., no enzyme present) were typically less than 5% of the radioactivity recovered as ceramides. Using this procedure, recovery of the ceramides was quantitative.

The final concentration of the alkylamines in the reaction mixtures were determined by the fluorescamine procedure [30] standardized for each alkylamine: the apparent inhibition constants for these compounds were reproducible only within a 2- to 3-fold range, presumably due to their poor solubility and/or adsorption to the test tubes. When the apparent inhibitory constants were based on the quantity of the alkylamine added, rather than the concentrations in the reaction mixtures determined by fluorescamine, the values were not reproducible and the Lineweaver-Burk plots were nonlinear.

The following terminology is used throughout; competitive (K_{is} = finite, $K_{ii} = \infty$), and mixed (K_{is} and K_{ii} = finite) inhibition. All kinetic constants were apparent values (K_{is}app, K_{ii}app, etc.). For the more potent inhibitors, $K_i$app values were determined with several sub-

strate concentrations (0.1 to 8 K_m) from Lineweaver-Burk plots and the appropriate slope (for $K_{is}app$) and 1/v-intercept (for $K_{ii}app$) replots [31] which were evaluated by the least-squares method.

For convenience, the $K_{is}app$ and $K_{ii}app$ values for less potent inhibitors were determined from the equations

$$K_{is}app = \frac{[I]}{\frac{\text{slope (I)}}{\text{slope (0)}} - 1} \qquad K_{ii}app = \frac{[I]}{\frac{\text{intercept (I)}}{\text{intercept (0)}} - 1}$$

where slope (__) or intercept (__) were from Lineweaver-Burk plots in the presence (I), or absence (0) of the inhibitor using 4MU-Glc or NBD-C_{12}-GC as substrates. $K_{is}app$ and $K_{ii}app$ values of the potent inhibitors were within ±20% by either method and with either substrate for representative inhibitors. IC_{50} values (the concentration of inhibitor which resulted in 50% inhibition) were determined with 4MU-Glc (4 mM) as substrate. All inhibitors were rapidly reversible and full recoveries of enzymatic activities were obtained in dilution experiments. The values represent the mean of at least three experiments conducted in duplicate. $V_{max}app$ values were reproducible within ± 7%. All plots and replots were linear with correlation coefficients greater than 0.990. Comparative inhibitor studies with the normal and Type 1 AJGD enzymes were conducted in matched sets with exactly equal incubation times.

Kinetic Studies in the Presence of "Co-Glucosidase" and/or Low Detergent Concentrations. Human "co-glucosidase" was purified from fresh spleen [8] obtained at surgery of an 8 y.o. male with idiopathic

thrombocytopenia purpura. This preparation also was delipidated with 20% n-butanol/isopropyl ether (1:1; v/v) at 4°C. The final preparation had one contaminant protein band ($M_r \gtrsim 53,000$) visualized with Coomassie blue after SDS-PAGE (polyacrylamide gel electrophoresis). As previously reported [8], no protein bands were stained with Coomassie blue at the expected M_r (=6,000) of "co-glucosidase". However, a single intense band at M_r = 6,200 was visualized with antibody to bovine "co-glucosidase" after electroblotting of the protein from 12% SDS-PAGE gels onto nitrocellulose filters. Using native PAGE, similar bands to those reported for bovine "co-glucosidase" [8] were visualized with Stains-All. Anti-bovine "co-glucosidase" antiserum was a generous gift from Dr. Norman S. Radin.

Studies of the effects of "co-glucosidase", low detergent concentrations and/or phosphatidylserine on the $K_i$app values of the N-C_n--dNMs or GS derivatives were conducted in polyethylene tubes to avoid adsorption of the inhibitors or enzyme. Pilot studies using Teflon™ tubes gave the same results as those obtained in polyethylene tubes. For these studies, all lipoidal compounds were added to dry tubes in 1-3 µl of chloroform/methanol (2:1; v/v) and immediately diluted to 150 µl with Buffer A (0.05 M phosphate/0.04 M citrate, pH 5.5, containing 4 mM β-mercaptoethanol, 1.25 mM EDTA) and substrate (4 mM 4MU-Glc). The reactions were initiated by the addition of enzyme and terminated, after 0.25 to 0.5 h at 37°C. Control tubes contained Buffer A, 1-3 µl chloroform/methanol (2:1; v/v) and enzyme; this amount chloroform/methanol had no effect on enzymatic activity. For studies in the absence of all detergents, IC_{50} values, instead of $K_i$app values, were determined with 4 mM 4MU-Glc and varying concentrations of the inhibi-

tors, since 1) 50 to 100 times more enzyme was required than in the presence of detergents, 2) reproducible $K_m$app values could not be obtained at < 0.1 mM Triton X-100 or < 0.3 mM taurocholate in the absence of Triton X-100. In the absence of Triton X-100 and negatively charged lipids, "K_m" values ranged from "20 to 100 mM" which was in excess of the solubility of this substrate and 3) reasonably linear (i.e., correlation coefficients $\geq$ 0.990) Lineweaver-Burk plots could not be obtained at very low taurocholate or phosphatidylserine concentrations (< 0.3 mM) in the absence of Triton X-100 or in the absence of all detergents and negatively charged lipids. K_{is}app values were reliably determined in the presence of 0.5 to 8.0 mM taurocholate in the absence of Triton X-100. Furthermore, in the absence of Triton X-100 and negatively charged lipids, the enzyme activity was stable for about 30 min, and the hydrolytic rates were reproducible within ± 30-40% with 4 mM 4MU-Glc. The IC_{50} values were the average of six determinations and were accurate within about a 2- to 3-fold range. The diluted pure normal enzyme used for these studies also contained about 0.3% ethylene glycol.

Results

Interaction of Acid β-Glucosidase with Alkylglycons and Alkylamines. The $K_i$app values for various alkylglycon and alkylamine inhibitors of the normal and Type 1 AJGD enzymes are shown in Table 9. Enzyme activities were determined in the presence of 4 mM Triton X-100 and 4.65 mM taurocholate at pH 5.75, primarily with 4MU-Glc as substrate and with NBD-C_{12}-GC as substrate for representative compounds;

TABLE 9

Apparent Inhibition Constants for Inhibitors of the Normal and Type 1 Ashkenazi Jewish Gaucher Disease Acid β-Glucosidases (37°C, pH 5.75)

Inhibitor[a]	K_i (Normal)	K_i (Type 1 AJGD)	$\frac{K_i \text{ (Type 1 AJGD)}}{K_i \text{ (Normal)}}$
Alkylglucosides[b]:	(mM)	(mM)	
C_0	220 ± 50	300 ± 64	1.4
C_1	298	441	1.47
C_6	3.3 ± 0.1	8.62	2.61
C_7	1.2 ± 0.1	6.4	5.3
C_8	0.32 ± 0.04	2.2	6.9
C_9	0.17 ± 0.03	1.1	6.5
C_{10}	0.16 ± 0.02	0.7	4.4
C_{12}	0.15 ± 0.04	0.6	4.13
Alkylamines[c]:	(mM)	(mM)	
C_3	50		
C_6	1.0 - 1.7		
C_7	0.7 - 1.4		
C_8	0.31 - 0.43	0.9	2.4
C_9	0.22 - 0.35		
C_{10}	0.11 - 0.2	0.4	2.6
C_{12}	0.1 - 0.17	0.3	2.2
C_{14}	0.090 - 0.16	0.3	2.4
C_{18}	0.08 - 0.14	0.2	1.81
N-C_n-dNMs[b]:	(μM)	(μM)	
C_0	53 ± 8	560 ± 90	10.6
C_1	35	140	4
C_4	190	470	2.5
C_{10}	0.10 ± 0.05 (0.03 - 0.05)[d]	0.51 ± 0.02 (0.75 - 1.3)[d]	5.1
C_{12}	0.04 ± 0.01 (0.02 - 0.05)[d]	0.45 ± 0.02 (0.56 - 1.2)[d]	11.3
C_{14}	0.09 ± 0.01 (0.03 - 0.04)[d]	0.44 ± 0.03 (0.56)[d]	4.9
Sphingosines:	(μM)	(μM)	
Sphingosine	29 ± 9[b]	182 ± 30[b]	6.3
GS	20 ± 5[c]	120 ± 20[c]	6.0
N-hexyl-GS	0.20 ± 0.05[c] (0.11 - 0.38)[d]	1.4 ± 0.3[c] (4.1)[d]	7.0

[a] Determined in 4 mM Triton X-100/4.65 mM taurocholate,
[b] K_{is}app value,
[c] $K_i$app ($K_{ii} = K_{is}$) value, [d] calculated values.

no differences in the pattern of inhibition or $K_i$app values were observed with either substrate. For the normal or Type 1 AJGD enzymes, the alkylglycons were mixed inhibitors with K_{ii}app = 2 to 10 K_{is}app (i.e., predominantly competitive), while the alkylamines were noncompetitive (i.e., K_{ii}app = K_{is}app).

For the series of 1-O-C_n-β-glucoses and N-C_n-dNMs or the alkylamines, the K_{is}app or $K_i$app values, respectively, for the normal enzyme were related to the alkyl chain length. These relationships for the alkylglycons, shown as -ln[K_{is}app(alkylglycon)/K_{is}app(β-glucose)] vs. alkyl chain length (Fig. 23A), were biphasic. The K_{is}app values with methyl-β-glucoside or N-C_1- or N-C_4-dNM, were greater than or equal to those of the parent glycons (Table 9). With longer alkyl chains (C_{10}-, C_{12}- and C_{14}-), the inhibitory potencies were increased by about three orders of magnitude and approached a limit at an alkyl chain length of 14 carbons. The curve describing the K_{is}app values [referenced to K_{is}app (β-glucose)] for the N-C_n-dNMs paralleled that for the alkyl-β-glucosides but at about three orders of magnitude lower values. When referenced to the K_{is}app for dNM, the N-C_n-dNM curves nearly overlapped those for the alkyl-β-glucosides. With the Type 1 AJGD enzyme, the K_{is}app values for the alkylglycon derivatives were about 5 to 7 times greater than the respective normal values (Table 9). This difference was not significant with 1-O-methyl-β-glucose and was about 2-fold with the N-C_4-dNM.

For the normal or Type 1 AJGD enzymes, the K_{is}app values (37°C, pH 5.75) for the N-C_n-dNMs agreed, within a factor of 2 to 4, with those calculated (Table 9) from the free energy equation:

Figure 23

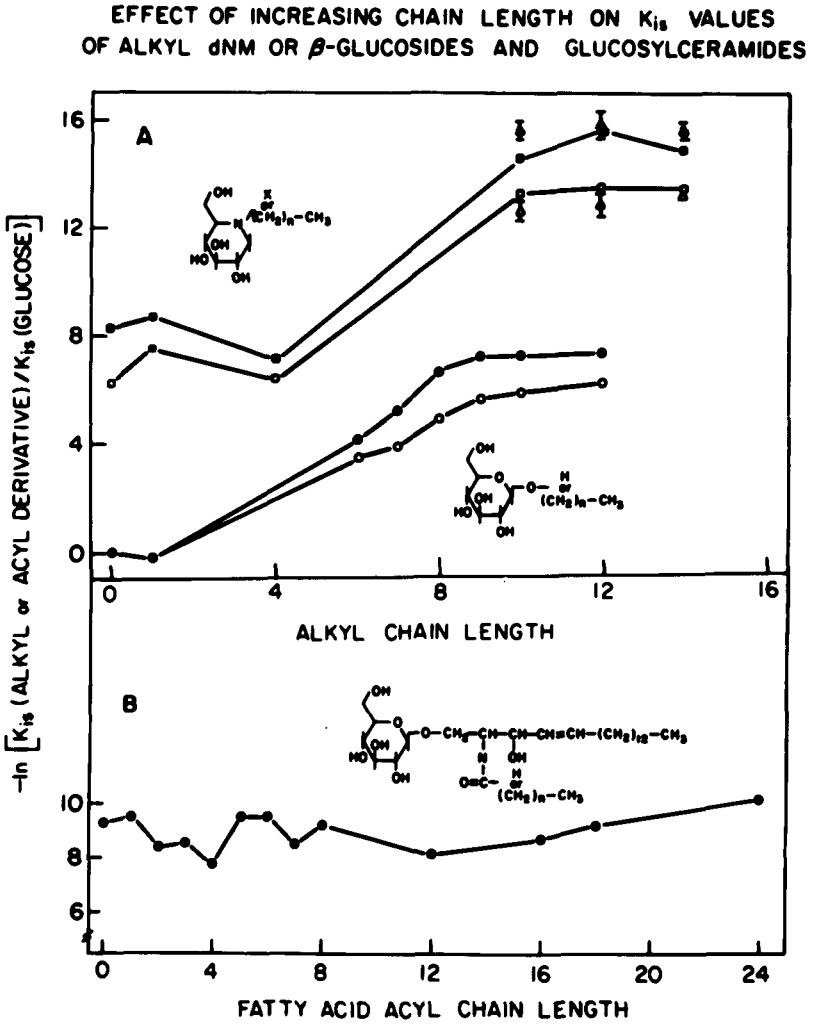

EFFECT OF INCREASING CHAIN LENGTH ON K_{is} VALUES
OF ALKYL dNM OR β-GLUCOSIDES AND GLUCOSYLCERAMIDES
A
B
$-\ln [K_{is}(\text{ALKYL or ACYL DERIVATIVE})/K_{is}(\text{GLUCOSE})]$
ALKYL CHAIN LENGTH
FATTY ACID ACYL CHAIN LENGTH
0
4
8
12
16
20
24
6
10

Figure 23:

Effect of increasing alkyl (A) or fatty acid acyl (B) chain length on the K_{is}app values for N-C_n-dNM (■,□) or alkyl-β-glucosides (●,○) in A and C_n-GCs in B. The first point of the C_n-GC curves (B) is for GS, i.e. deacylated GC. Closed and open symbols are for the normal and Type 1 AJGD enzymes, respectively. The values are plotted as the additional free energy ($\Delta\Delta G^\circ$) of binding relative to that for β-glucose (eq 1). In (A) the triangles represent the predicted K_{is} values calculated from eq 1 and the data in Table 9. 4MU-Glc was the substrate. The X in (A) near the N of dNM represents the absence of an H^+ group, i.e., unprotonated dNM [14].

$$\Delta\Delta G^{\circ} = -RT \left[\ln \frac{K_{is}app(dNM)}{K_{is}app(glucose)} + \ln \frac{K_{is}app(alkyl\text{-}\beta\text{-}glucoside)}{K_{is}app(glucose)} \right] \quad (1)$$

where $\Delta\Delta G^{\circ}$ represents the additional free energy of binding, relative to β-glucose.

Using splenic sphingosine, a natural alkylamine which is predominantly d18:1 [32], mixed inhibition was obtained with $K_{is}app = 29 \pm 9$ μM and $K_{ii}app = 185 \pm 30$ μM (Table 9). In comparison, the $K_i app$ value for the saturated chain, octadecanamine, was about 3-fold greater (100 μM) than the $K_{is}app$ value and similar to the $K_{ii}app$ value for sphingosine. The addition of a β-glucosyl moiety to sphingosine [i.e., glucosylsphingosine (GS)], resulted in a small decrease in $K_i app$ ($K_{ii} = K_{is}$) = 20 μM and the inhibition was noncompetitive [also, 7]. More potent noncompetitive inhibition ($K_i app = 0.3$ μM) was observed with N-hexyl-GS [also, 7]. The latter $K_i app$ value was in agreement with that obtained by Erickson and Radin (33; 0.33 μM), although they observed competitive inhibition under different assay conditions. Using the $K_{is}app$ values for 1-O-hexyl-β-glucoside (3.3 mM) and sphingosine (0.029 mM) and assuming independent additivity of binding energies, the calculated $K_i app$ for N-hexyl-GS (0.38 μM; eq 1) agreed well with the observed normal values. Similarly, using the $K_i app$ for GS and the $K_i app$ for N-hexanamine, the calculated $K_i app$ for N-hexyl-GS was 0.11 μM for the normal enzyme. Similar results were obtained for the Type 1 AJGD enzyme but at 5- to 7-fold greater K_i values (Table 9).

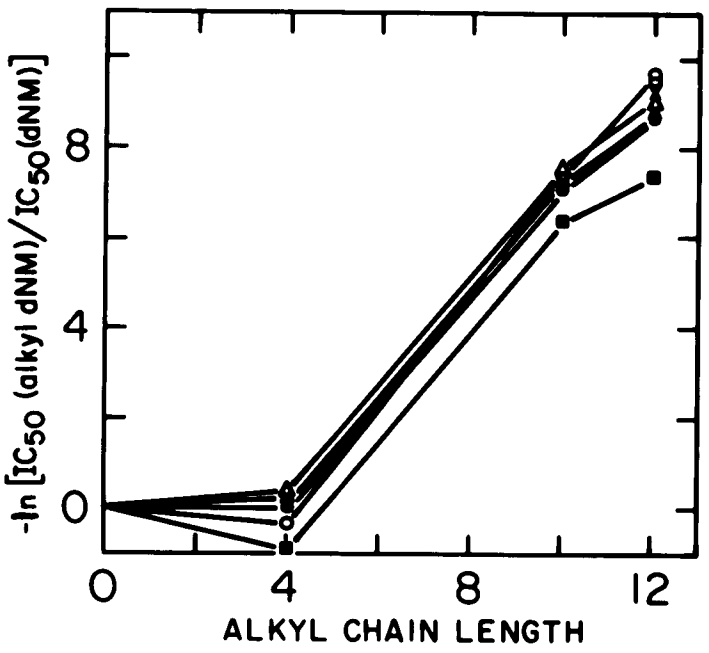
The biphasic nature of the $K_{is}app$ curves as a function of alkyl chain length for the dNM and β-glucoside derivatives, the apparent additivity of $\Delta\Delta G^{\circ}$ for several inhibitors and the protection of the

enzyme by N-C_{10}-dNM and GS from inactivation by conduritol B epoxide [14] suggested an optimal chain length for interactions of lipoidal inhibitors with distinct domains of the acid β-glucosidase active site. Since some of these effects could result from amphiphile interactions with the enyzme and/or lipoidal inhibitors, varying assay conditions were used to evaluate some of these potential interactions.

Interactions of Enzyme, Inhibitors, Amphiphiles and "Co-Glucosidase. For the normal enzyme and the series of N-alkyl-dNM derivatives, a biphasic variation of IC_{50} with increasing alkyl chain length was obtained under several different assay conditions (Fig. 24, Table 10), i.e., in the absence or presence of taurocholate (below or above the critical micellar concentration [CMC] = 3.5 to 4 mM [33]) or Triton X-100 < CMC ($\simeq$ 0.24 mM [34]). The results are shown as -ln[IC_{50}(alkyl-dNM)/IC_{50}(dNM] for direct comparison to the results in Figure 23A. The IC_{50} (dNM) was about 3.2 or 4 orders of magnitude greater than the IC_{50} (C_{10}-dNM) or IC_{50} (C_{12}-dNM), respectively. The curve obtained in the presence of 4 mM Triton X-100 and 4.65 mM taurocholate was parallel to, but lower (3-to 5-fold) than, those obtained in the absence of 4 mM Triton X-100 (Fig. 24). The concentration of taurocholate in the absence of Triton X-100 had little effect on the shape of the IC_{50} curves or the K_{is}app values for these inhibitors.

The effects on the IC_{50} values for inhibitors by increasingly complex mixtures of amphiphiles and/or "co-glucosidase" allowed for some insight into the interactions of the lipoidal compounds. Varying amounts of amphiphiles in the presence or absence of "co-glucosidase"

Figure 24



Effect of increasing alkyl chain length on the IC_{50} values for N-alkyl-dNM in the absence (O) or presence of various concentrations of either Triton X-100 or taurocholate. Taurocholate concentrations were 0.5 (Δ), 1.0 (▲) or 8 (●) mM. IC_{50} values for dNM and N-C_{12}-dNM were determined in the presence of 0.05 (□) and 0.10 (X) mM Triton X-100. The curve described by (■) was with 4.0 mM Triton/4.65 mM taurocholate.

TABLE 10

Effect of Taurocholate on the $K_{is}app$ Values of N-Alkyl-dNMs with Normal Acid β-Glucosidase (37°C; pH 5.75)

Inhibitor	Taurocholate[a] 0.5 (mM)	1.0 (mM)	8.0 (mM)
	$K_{is}app$[b] (μM)		
N-C_n-dNM:			
C_0	111	94	81
C_4	72	75	57
C_{10}	0.10	0.081	0.067
C_{12}	0.017	0.017	0.016

[a] Assays conducted in the absence of Triton X-100 with 4MU-Glc as substrate,
[b] Values were reproducible within ± 30%.

had no effect on IC_{50} (dNM) (Table 11). In comparison, increasing the concentration of Triton X-100, above the CMC, increased the IC_{50} (C_{12}--dNM) by an order of magnitude. In the presence of 0.8 mM Triton X-100 (with or without "co-glucosidase"), 0.5 mM phosphatidylserine increased the IC_{50} (C_{12}-dNM) value about 4-fold; smaller effects on the IC_{50} (GS) values were observed (Table 11). Increasing the Triton X-100 concentration from 0.8 mM to 4 mM in the presence or absence of phospha tidylserine and/or "co-glucosidase" increased the IC_{50} (N-hexyl-GS) value about 10-fold. In the presence of 0.8 mM Triton X-100, "co-glucosidase" had no effect on the IC_{50} (N-hexyl-GS) value. With several different assay conditions, the IC_{50} (N-hexyl-GS) values were proportional to the calculated Triton X-100 micelle concentration, assuming an aggregation number of 150 for Triton X-100 micellization (i.e., 0.8 mM Triton X-100 $\simeq$ 3.7 μM Triton X-100 micelles and 4 mM Triton X-100 $\simeq$ 25 μM Triton X-100 micelles).

Multiple Inhibition Analysis. The linearity of the 1/v-intercept or slope vs. [I] replots for the alkyl -amine, -β-glucoside or -dNM and sphingosyl derivatives in the presence of 4 mM Triton X-100 with or without 4.65 mM taurocholate suggested that these inhibitors each bound to single sites on the enzyme which affected enzymatic activity. In an effort to gain supporting evidence for the interaction of the alkylglycons or GS derivatives to a single active site (but to different active site domains), multiple inhibition studies with two different inhibitors were conducted using the normal enzyme. For these studies, Dixon plots were developed at a fixed concentration of the 4MU-Glc substrate (4 mM) and varying concentrations of each of two

TABLE 11

Effect of Amphiphiles and "Co-Glucosidase" on the IC_{50} Values for Inhibitors of Normal Acid β-Glucosidase (37°C, pH 5.5)

Amphiphile	IC_{50} Value			
	GS	N-Hexyl GS	dNM	N-C_{12}-dNM
	(μM)	(μM)	(mM)	(μM)
No Amphiphile			0.3	∿ 0.02
Triton X-100 (mM):				
0.05			0.4	∿ 0.02
0.10			0.5	∿ 0.02
0.8	5	0.02	0.5	0.26
4.0	7.5	0.20	0.4	0.25
Phosphatidylserine (0.5 mM):				
+ 0.8 mM Triton X-100	12.5	0.03	0.3	1.0
+ 4.0 mM Triton X-100	15	0.3	0.4	
+ 0.8 mM Triton X-100 + 4 mg "co-glucosidase"	7	0.08	0.2	0.9
"Co-Glucosidase" (4 μg):				
+ 0.8 mM Triton X-100	5	0.02	0.4	0.2

inhibitors. The assays were conducted in buffer A, pH 5.5, containing only 4 mM Triton X-100. Table 12 summarizes the results with mixtures of two different inhibitors. Within experimental error, the interaction between each set of two different inhibitors gave parallel Dixon plots (Fig. 25), i.e., the inhibitors were mutually exclusive.

Interaction of Acid β-Glucosidase with C_n-GC Derivatives. Using the assay system containing the 4 mM Triton X-100/4.65 mM taurocholate, the C_n-GC derivatives were competitive inhibitors of 4MU-Glc or NBD-C_{12}-GC hydrolysis by the normal enzyme. In comparison to the alkyl-glycons or N-hexyl-GS, the increasing length of the fatty acid acyl chain (C_1 to C_{24}) had small (less than 10-fold) irregular effects on the K_{is}app values (Table 13). The respective K_{is}app values for representative C_n-GCs were increased up to 2-fold when NBD-C_{12}-GC was used as substrate, but the results were somewhat more variable than obtained with 4MU-Glc as substrate (Table 13). The 2-N-stearyl- and 2-N-lignoceryl-dihydrosphingosyl-1-O-β-glucosides had K_{is}app values about two orders of magnitude greater than the respective unsaturated GCs, confirming the observation of Vaccaro et al. [35]. The $K_m$app values for the hydrolysis of the representative C_n-GCs were similar (within about 2-fold) to the respective K_{is}app values (Table 13).

With the Type 1 AJGD enzyme, the effects of increasing GC fatty acid acyl chain length were the same as the normal enzyme and K_m(AJGD) = K_m(normal) for the respective C_n-GCs. The equality of the $K_m$app values for the AJGD and normal enzymes was maintained under varying assay conditions, i.e., Triton X-100, 1.30 to 8 mM, and taurocholate, 0 to 16 mM, or phosphatidylserine, 0 to 2 mM (data not shown).

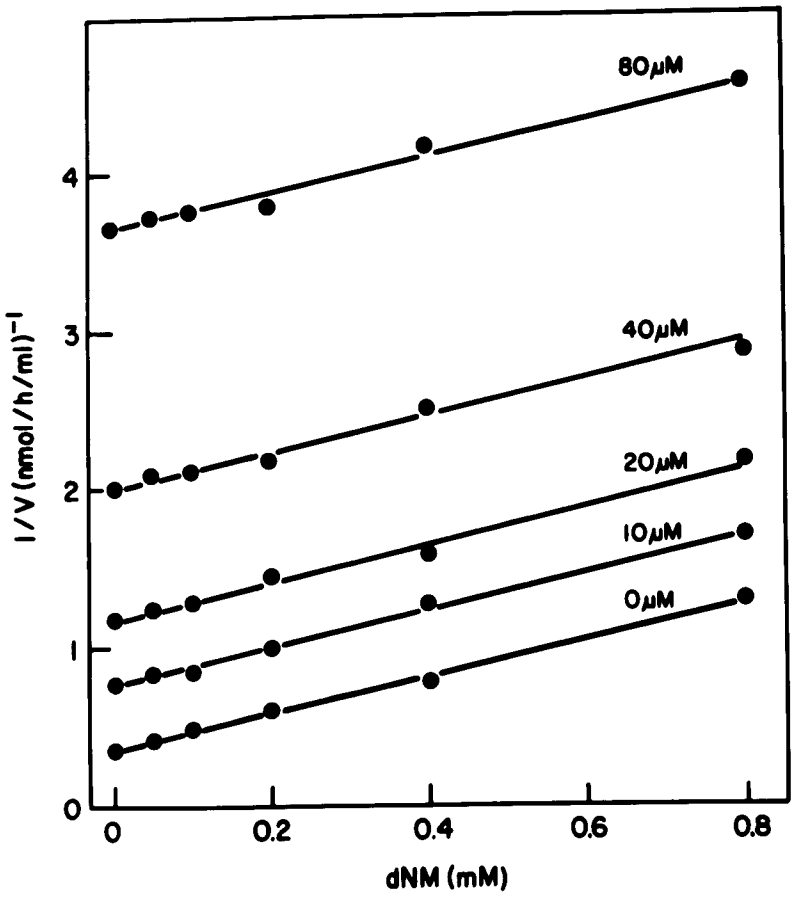
TABLE 12

Multiple Inhibition Analyses of Normal Acid β-Glucosidase[a]

Inhibitor[a]	6-Glucono-lactone(C)[b]	dNM (M)	N-Dodecyl-dNM(M)	GS (NC)	Sphingo-sine(M)
6-Gluconolactone	--	ME[c]	ME	ME	ME
dNM	--	--	ME	ME	ME
N-dodecyl-dNM	--	--	--	ME	ME
GS	--	--	--	--	ME

[a] Assays conducted in 4 mM Triton X-100 at pH 5.50,
[b] Type of inhibition observed under these experimental conditions; C = competitive (K_{is}app = finite, K_{ii}app = ∞), M = mixed inhibition (K_{ii}app = 4 to 9 K_{is}app, i.e., predominantly competitive), NC = noncompetitive (K_{is}app = K_{ii}app); see Osiecki-Newman et al., 1986,
[c] ME = mutually exclusive.

Figure 25



Dixon plot of 4MU-Glc hydrolysis by the normal acid β-glucosidase in the presence of dNM and GS (pH 5.50, 37°C), demonstrating a typical mutual exclusion pattern (see Table 11).
The ordinate was multiplied by 10^5.

TABLE 13

Apparent Kinetic Constants for Glucosyl Ceramide Derivatives with the Normal and Type 1 AJGD Acid β-Glucosidases (37°C; pH 5.75)

	Normal				Type 1 AJGD	
			K_{is} (μM)			
C_n-GC[a]	V_{max}[b]	K_m (μM)	4MU-Glc[c]	NBD-C_{12}-GC	V_{max}	K_m (μM)
C_1[c]	0.19	14 ± 6	16 ± 5	49 ± 10	0.005	15 ± 4
C_2	0.42	32 ± 8	48 ± 7		0.012	18 ± 5
C_3	0.62	20 ± 4	40 ± 10	50 ± 7	0.016	35 ± 5
C_4	1.26	42 ± 10	91 ± 20	99 ± 21	0.033	47 ± 12
C_5			16 ± 4			
C_6			17 ± 5	37 ± 9		
NBD-C_6	1.22	50 ± 20	40 ± 10		0.034	50 ± 30
C_7			45 ± 3			
C_8	2.50	33 ± 9	20 ± 5	39 ± 10	0.048	35 ± 10
C_{12}			73 ± 6	109 ± 16		
NBD-C_{12}	0.81	30 ± 10	78 ± 10		0.023	30 ± 10
C_{16}	2.00	34 ± 5	35 ± 10		0.030	36 ± 5
C_{18}	1.38	28 ± 5	21 ± 3	43 ± 11	0.017	31 ± 5
C_{24}			8 ± 2			
C_{18}-DihydroS[d]			> 800			
C_{24}-DihydroS			> 1000			

[a] C_n-GC is N-acyl-sphingosyl-1-O-β-glucoside with the acyl chain of n carbons in length, e.g., C_{16} is the palmityl-GC.

[b] V_{max} = μmol/min/ml.

[c] Substrate as defined in text.

[d] DihydroS is dihydrosphingosyl-1-O-β-D-glucoside.

The fatty acid acyl chain length had a pronounced effect on the hydrolytic rates of the C_n-GCs by either enzyme source (Table 13). For the normal enzyme, the V_{max}app values increased by 13-fold with the fatty acid acyl chain length increasing from C_1 to C_8. The C_{16}- and C_{18}-GC substrates had V_{max}app values of 80% to 55% of that for the C_8 derivative. The NBD-C_6- and NBD-C_{12}-GCs had lower V_{max}app values than might be expected from the V_{max}app of C_8-GC, suggesting that the fluorescent group interfered with catalysis. The V_{max}app values obtained with the 7500-fold enriched Type 1 AJGD acid β-glucosidase indicated that the shorter chain C_n-GCs (n = 1 to 8) were hydrolyzed at nearly the same relative rate [(V_{max}app (NBD-C_{12}-GC)/V_{max}app (C_n-GC)] as with the normal enzyme, whereas the longer chain C_n-GCs (n = 16 to 18) were hydrolyzed at somewhat slower relative rates.

In comparison to the C_n-GCs and alkylglycons, the alternate substrates, para-, ortho-, and meta- nitrophenyl-β-D-glucosides, had IC_{50} values with either the normal or mutant enzyme of 3.0, 8.5 and 7.4 mM, respectively. The 1- or 2-napthyl-β-D-glucosides had IC_{50} values > 25 mM.

Discussion

Several alternative explanations could account for the influence of increasing chain length on the apparent kinetic constants for inhibitors and substrates: 1) the enzyme's active site contains two hydrophobic domains, the aglycon binding site and the "third" domain, each equivalent to multiple "subsites" of unit carbon bond length, which can accommodate only chain lengths shorter than the acyl and

sphingosyl chains of GC, i.e., hydrophobic chains of greater than about 16 carbon-carbon bond lengths would have no additional effects on the binding of substrates or inhibitors, 2) a hydrophobic domain of the active site ($\geq$ 17 carbon-carbon bond lengths) competes with detergent micelles for binding of alkylglycons up to a given chain length after which no increase would be observed due to the greater energies required to extract the compound from the micelles i.e., the apparent binding and rate constants were composites which reflected interactions with the active site as well as detergents, or 3) a combination of 1 or 2 above and the presence of a relatively hydrophilic region within the enzymes' active site for interaction with the hydroxyl and amine groups of the sphingosyl moiety of GC. The present studies provide support for the third explanation.

Previous studies and the present results indicated that increasing concentrations of Triton X-100 above the CMC inhibited acid β-glucosidase activity and directly increased the $K_m$app for GC [7] and the $K_i$app or IC_{50} values for N-hexyl-GS (Tables 9 and 10). Legler and Liedtke [21], using the bovine β-glucosidase, found similar effects of Triton X-100 with the 4-C_9- and 4-C_{11}-umbelliferyl-β-glucoside substrates. These findings and the same relative magnitudes of the $K_m$app values for the longer alkylumbelliferylglucosides observed in assay systems containing Triton X-100 [13,21], indicated that while Triton X-100 may interact with the enzyme, it acts primarily as a solubilizer and diluant for lipoidal inhibitors and substrates [7]. However, in the presence of only taurocholate, a negatively charged lipid which forms small aggregates (n = 4-5; [33]), the $K_m$app values for the 4-C_9-

- or 4-C_{11}-umbelliferyl-β-glucosides [21] and the K_{is}app for N-C_n-dNMs (Table 10) decreased as a function of chain length. These results suggested the presence of a "hydrophobic" domain within the active site of the bovine and human enzymes. However, the IC_{50} values for the N-C_{10}- and N-C_{12}-dNMs were about 3-to 5-fold greater in the presence of Triton X-100 concentrations $\geq$ CMC, whereas the IC_{50}(dNM) was unaltered (Fig. 24). These results indicated that the K_{is}app values obtained in Triton X-100 systems ($\geq$ CMC) contain terms which reflect the binding of the glycon and aglycon moieties of these inhibitors to the enzyme and the energy required to extract increasingly hydrophobic compounds from the micelles. Furthermore, the decreased potency of inhibition by N-C_{12}-dNM in the presence of phosphatidylserine, with or without "co-glucosidase" (Table 11), indicates additional interactions between this inhibitor and lipid in Triton-based systems. The above results indicate that the length (in carbon bonds) of a hydrophobic domain in this enzyme's active site cannot be ascertained with certainty in detergent or lipoidal systems. The unreliability of the quantitative kinetic data (e.g., "K_m" > 200-100 mM) in the absence of Triton X1000 or negatively-charged lipids limited their usefulness. But, the fact that similar patterns of effect of alkyl chain length for N-C_n-dNMs were obtained in the presence or absence of detergent or taurocholate indicated the hydrophobic domain in the active site may be long enough to accommodate C_{12} to C_{14} chains.

The above discussion assumes comparable inhibition patterns and stoichiometry of the inhibitors with the enzyme in the presence of variable concentrations and mixtures of amphiphiles. The linearity of the appropriate replots of slopes or 1/v-intercepts vs. [I] and the

predominantly competitive inhibition for dNM and N-C_n-dNMs in the presence of Triton X-100, with or without "co-glucosidase" or taurocholate, indicates a 1:1 (mole/mole) stoichiometry between these inhibitors and the enzyme's active site [36]. The presence of a single active site on acid β-glucosidase [6,13], the protection afforded by N-C_n-dNMs or GS to the enzyme from inactivation by conduritol B epoxide and the mutual exclusion of glycon, alkylglycon and sphingosyl derivatives indicated interactions of these inhibitors at the active site.

Using several different assay systems, the apparent affinity of sphingosine, GS and C_n-GCs (Tables 9 and 13) for the normal acid β-glucosidase were the same, and the dihydro-ceramide β-glucosides had 40- to 50-fold increased K_{is}app values (Table 13; [35,37]). These findings and the biphasic nature of the K_{is}app vs. N-C_n-dNM curves (Figs. 23 and 24) indicated the presence of a relatively hydrophilic region of 4 to 5 carbon bonds in length within the active site for interaction with the amine, the hydroxyl, and possibly, the double bond, of the sphingosyl moiety (Fig. 26). The protection provided by sphingosine or GS to acid β-glucosidase from inactivation by conduritol B epoxide [11,14], the apparent mutual exclusivity of several glycons and sphingosine or GS (Table 12) and the relatively low apparent affinity of nitrophenyl-, naphthyl-and 4MU- β-glucosides, provided additional support for the existence of the hydrophilic region and indicated that the interactions with this region were somewhat specific. The specificity of the proposed (relatively) hydrophilic region also was indicated by: 1) erythro-N-acetyl-GC in liposomes was bound and hydrolyzed better by acid β-glucosidase than the threo derivative [37], 2) octyl-β-glucoside [7] and GS [38,39] were poor substrates, and

3) the $K_m$app for 3-keto-ceramide-β-glucoside was increased relative to the respective GC [37]. That this hydrophilic region in the acid β-glucosidase active site also interacted with the fatty acid acyl chain of GC was indicated by the increasing inhibitory potency of short chain ($n \leq 6$) N-C_n-GS derivatives [33], the two orders of magnitude decreased apparent affinty of the normal enzyme for hexanoyl-GC compared to N-hexyl-GS (Tables 9 and13), and the different apparent natures of the inhibition by C_6-GC and N-C_6-GS in our assay systems.

The studies with C_n-GCs indicated the existence of a second hydrophobic domain, the aglycon binding domain [7], within the acid β-glucosidase active site. Because these substrates were contained in Triton X-100 micelles, the $K_m$app or K_{is}app values would have been expected to increase as a function of fatty acid acyl chain length due to the increasing energy requirements for extraction of the C_n-GCs from the micelles. Indeed, such a trend was noted with longer chain N-C_n-GS ($n > 6$) [33]. The small irregular effects of increasing fatty acid acyl chain length on $K_m$app or K_{is}app of the C_n-GCs (Fig. 23B; Table 13) indicated these kinetic parameters were not modulated by the acyl moiety. However, the abrupt increase in V_{max}app observed with the C_1- to C_4-GCs and the largest V_{max}app with the C_8-GC indicated that the interaction of the fatty acid acyl chain with the enzyme was related to modulation of the hydrolytic rate. The lower than expected V_{max}app values but unaltered $K_m$app or K_{is}app observed with the NBD-GC derivatives support this proposal: i.e., the NBD group interfered with hydrolysis up to 12 carbon bond lengths away from the reactive center. The effects of increasing fatty acid acyl chain length on V_{max}app might be explained by a greater affinity of the Triton X-100/taurocholate mixed

micelle for longer chain C_n-ceramides leading to a more rapid removal of these products from the enzyme. However, this explanation implies a progressive increase in hydrolytic rate as a function of fatty acid acyl chain length. The nearly equal V_{max}app values with the C_4- and C_{18}-GCs (Table 13) indicated that this mechanism was unlikely.

Similar data and trends were obtained with the Type 1 AJGD enzyme, except that the K_{is}app values for the sphingosyl, alkylglycon and specific glycon [14] derivatives were 4-17 times greater than the respective normal values. Furthermore, the $K_i$app value for conduritol B epoxide, a covalent active site directed inhibitor of acid β-glucosidase, was increased to a similar extent [13]. For several of these compounds, this decreased potency of inhibition remained constant using a variety of assay conditions, indicating that the values for these apparent kinetic constants likely reflect a property of the mutant enzyme and not detergent or lipid interactions with the enzymes, substrates or inhibitors. Importantly, the respective parallel effects on K_{is}app or V_{max} of increasing alkylglycon or C_n-GC chain length with the normal or Type 1 AJGD enzymes indicated that the kinetic abnormality of the mutant enzymes were strictly related to the interaction near the glycon head group and that the interaction with alkyl or acyl groups were not altered.

Within the limitations of these kinetic data obtained in detergent systems, a structure for the acid β-glucosidase active site can be proposed (Fig. 26). We suggest that the active site of acid β-glucosidase consists of three domains: 1) a hydrophilic pocket, the glycon binding domain, for interaction with the glucoside head group [14] and 2) two hydrophobic domains, the aglycon binding domain and the "third"

Figure 26

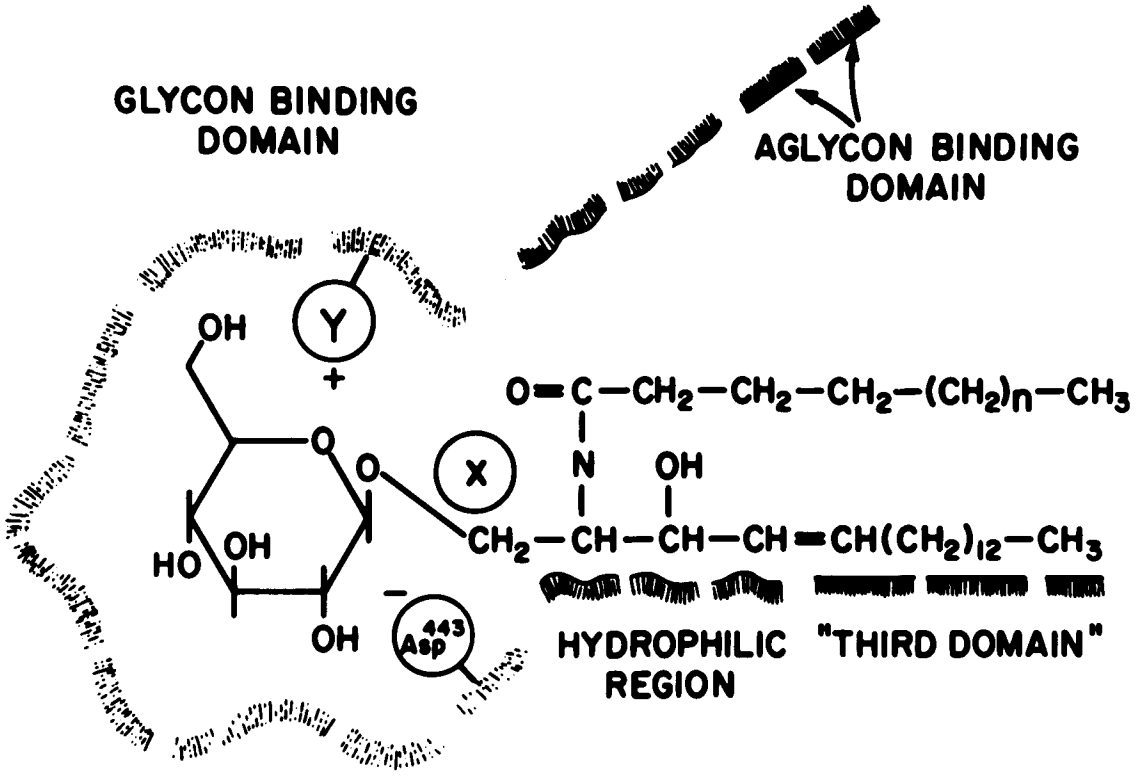

GLYCON BINDING DOMAIN
AGLYCON BINDING DOMAIN
OH
Y
+
O
O
X
HO
OH
OH
Asp 443
O=C—CH2—CH2—CH2—(CH2)n—CH3
N
OH
CH2—CH—CH—CH=CH(CH2)12—CH3
HYDROPHILIC REGION
"THIRD DOMAIN"

Figure 26:

A schematic representation of the proposed active site structure of normal or Type 1 AJGD acid β-glucosidase with bound C_n-GC. The glycon binding domain, a shared hydrophilic region of the three domains, and the two hydrophobic domains (the aglycon binding site and third domain) are indicated. The shared hydrophilic region includes the lower and upper wavy areas of about 4 to 5 carbon bonds in length. The lengths of the two hydrophobic domains are not specified. The outlines of each domain are discontinuous since no particular alignment of the amino acid sequence is implied. Asp^{443} is the catalytically necessary amino acid which participates in nucleophilic attack of the O-glucosidic bond. "Y" (cationic) and "X" are proposed amino acids for interaction with the ring oxygen of glucopyranose or the unprotonated N of nojirimycin (Osiecki-Newman et al., 1986). The domains and the proposed residues can have any orientation above, below or in the plane of the page. "X" is hypothesized to be altered in the Type 1 AJGD active site resulting in selectively decreased binding of unprotonated amines of specific inhibitors.

domain, for interaction with the fatty acid acyl chain and sphingosyl moieties of GC, respectively [7]. The lengths of these hydrophobic domains have not been defined, but the present studies indicate that they conform closely to the ceramide moiety of the natural substrates. These three domains also share a relatively hydrophilic region of 4 to 5 carbon-carbon bonds in length. Although the functions of the proposed active site domains within their natural environments are unknown, the "third" domain has been proposed to modulate k_{cat} [7]. The more extensive studies presented here indicate that, in this assay system, the "third domain" and aglycon binding domain modulate substrate affinity and hydrolytic rates, respectively. Recent studies have suggested the presence of other hydrophobic domains not in the active site for interaction with amphiphilic effectors [40].

Within the framework of this proposed model, the present studies suggest alternatives for the location of the single amino acid alteration(s) in the Type 1 AJGD acid β-glucosidase: 1) an amino acid substitution distant from the active site which leads to a conformational change in the enzyme, affecting the binding of particular inhibitors, or 2) an amino acid substitution in or near the active site affecting the interaction of inhibitors with specific residues. This substitution could directly involve a critical residue, X (Fig. 26), for binding or could result in local conformational changes affecting the position of this residue for inhibitor binding: the occurrence of such local effects has been reported recently in an elegant site-specific mutagenesis study of E. coli dihydrofolate reductase [41]. Based on the data and arguments presented here, the second alternative is more likely.

Chapter Eight

The Isolation of a Potential Membranous Segment of the Human Acid β-Glucosidase and Evidence for an "Active Core"

Materials and Methods

Materials

The following were obtained from commercial sources: (3-(Trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazirine ([^{125}I]-TID; Amersham, Arlington Hts., IL); Staphlococcus Aureus V_8 protease (V_8 protease; Miles Scientific, Naperville, IL); acetonitrile (ACN; American Burdick and Jackson Corp., Muskegon, MI); trifluoroacetic acid (TFA; Pierce Chemical Co., Rockford, IL); 4-methylumbelliferyl-β-D-glucopyranoside (4MU-Glc; RPI, Mount Prospect, IL); Vydac™ protein C-4 column (4.6 x250mm; The Sep/a/ra/tions Group, Hesperia, CA); Autofluor® (National Diagnostics, Somerville, NJ); Pronase® from Streptomyces Griseus (Pronase®; Calbiochem-Behring, Los Angeles, CA). [^{3}H]Br-CBE (8000 cpm/pmol) was the generous gift of Dr. Gunter Legler. The N-C_{10}-dNM-Sepharose was synthesized as described (1). All other reagents were the highest grade available.

Methods

[^{125}I]-TID Labeling and Cleavage Studies:

The pure β-Glc employed in these studies was prepared on the N-C_{10}-dNM-Sepharose column as described (1). The enzyme was labeled with [^{125}I]-TID using a modification of the photoactivation method of Brunner and Semenza (2): aliquots of the enzyme (0.45 nmol) were diluted to 0.1 M phosphate, pH 6.0, with a final concentration of 2.5 mM β-mercaptoethanol and 3 mM EDTA. [^{125}I]-TID (4.5 nmol) in ethanol was added and the reagent was activated by exposure to light at 350 nm

wavelength in a Farrand System 3 Spectrofluorometer (no slits) for 30 min at 22°C. An additional 4.5 nmol of the label was added to the incubation mixture and activated as above. The final ethanol concentration was < 5%. Labeling was demonstrated by analytical SDS-PAGE (3) and autoradiography of the dried gel.

The covalently labeled enzyme was digested with V_8 protease as previously described (4) and the resultant radiolabeled peptides were separated by reverse phase HPLC in a non-linear gradient of 0 - 80% ACN in 0.05% TFA. The peaks were detected by Abs^{280} and Abs^{214} and were collected in polypropylene tubes. Analytical SDS-PAGE (12.5%) and silver staining techniques (5) were used to assess the purity of the peaks and the presence of the label was demonstrated by autoradiography of the destained (5), dried gel.

Amino acid sequences were determined using gas-phase sequencing techniques (6) in the laboratory of Dr. Kenneth Williams, the Protein Chemistry Facility in the Dept. of Biophysics and Biochemistry, Yale University. Hydropathy profiles and secondary structural assignments were calculated using the method of Kyte and Doolittle (7) and the program of Corrigan and Huang (8), respectively.

Pronase® Cleavage Studies:

The pure β-Glc was incubated at 37°C with Pronase® in 0.1 M phosphate, pH 6.0, containing 1.4 mM β-mercaptoethanol and 1.75 mM EDTA. The ratio of Pronase® to enzyme protein was 5:1 (wt:wt). Enzyme activities were determined fluorometrically with 4MU-Glc as a substrate (9). β-Glc or Pronase® generated β-Glc peptides were labeled with [^{3}H]Br-CBE as previously described (4). Due to the large excess of

Pronase® required for proteolysis, cleavage was assessed by the loss of the protein band for β-Glc (Mr = 67,000) on SDS-PAGE. The presence of the label was determined by autoradiography for 30 days. Prior to autoradiography, the gels were soaked in Autofluor® for one hour and dried under vacuum and heat.

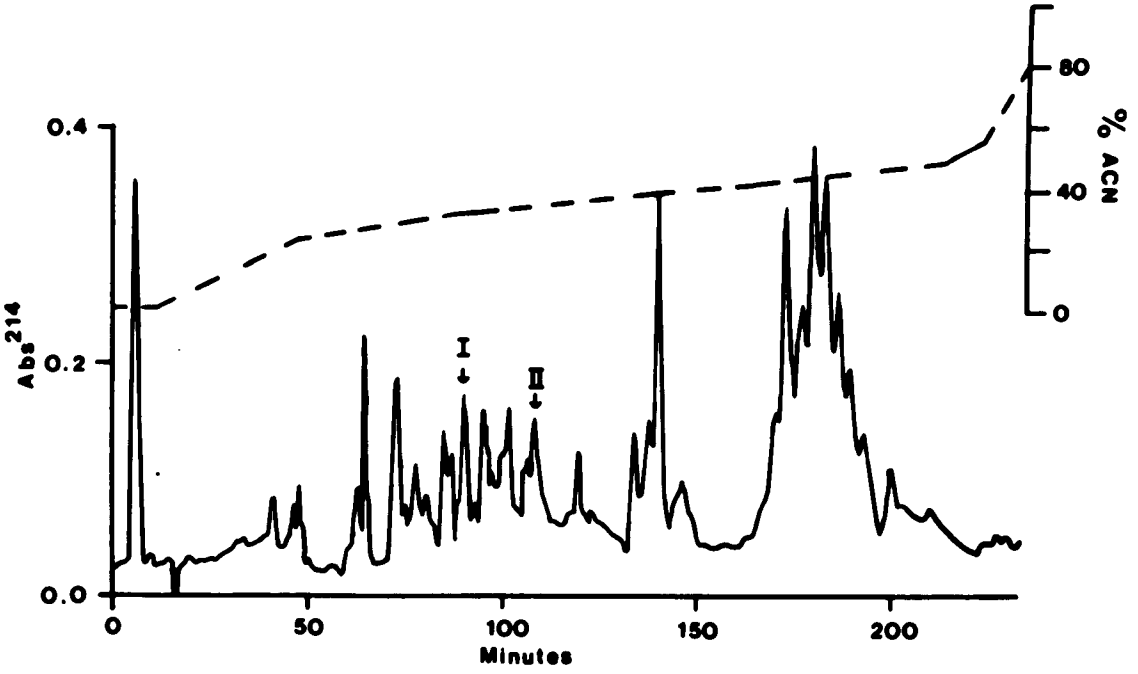
Results

Studies of a Potential Membranous Domain:

[^{125}I]-TID is a highly hydrophobic, photoactivated covalent label of membranes and membrane-associated regions of proteins (2). The photogenerated carbene is highly reactive and has been shown to interact with the membrane anchoring domains of a number of proteins, including acetylcholinesterase (10) and the sucrase/isomaltase complex (11). This reagent was used to isolate a potential membrane association domain of β-Glc. The covalent labeling of the pure enzyme was determined by demonstrating radioactivity (^{125}I) in the β-Glc protein band from SDS-PAGE (Mr = 67,000) (data not shown).

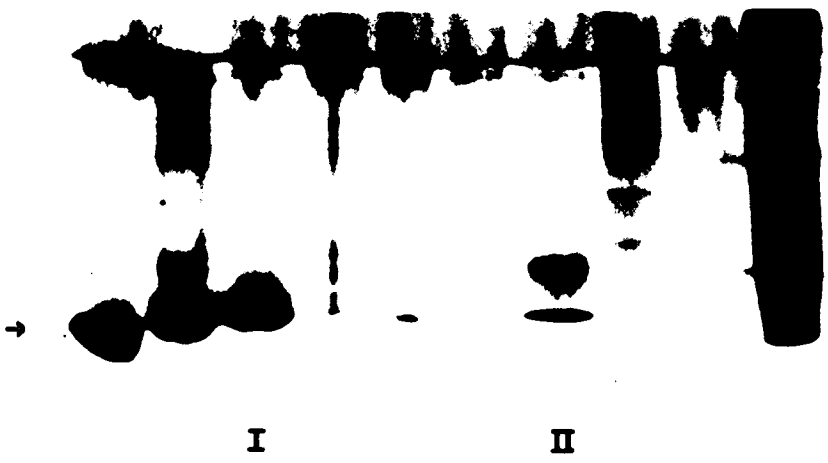
The radiolabeled enzyme was cleaved with V_8 protease and the resultant fragments were separated by reverse phase HPLC. Two peptides, I and II, were isolated. The elution profile is shown in Figure 27. The eluted peptides were analysed by SDS-PAGE using silver staining techniques to assess purity (Figure 28). The presence of the label on the peptides was determined by autoradiography (14 day exposure) of the destained, dried gel (Fig. 28) and is shown in Figure 29. Free [^{125}I]-TID was located below the dye front on the gel and did not interfere with the identification of the labeled peptides. The

Figure 27



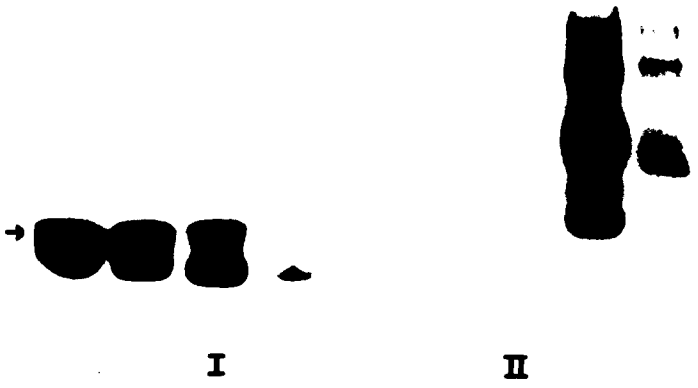
The elution profile of the [^{125}I]-TID labeled acid β-glucosidase after cleavage with V_8 protease from reverse phase HPLC as monitored by Abs^{214}. The gradient (0 - 80% ACN) is indicated above by the dashed line. Peptides I and II were eluted at 33.2 - 34.8% and 36.1 - 36.9% ACN, respectively.

Figure 28



SDS-PAGE of the peptides from V_8 protease cleaved [^{125}I]-TID labeled acid β-glucosidase after separation on reverse phase HPLC. The amino acid sequence of Peptide I is the N-terminus of the enzyme The protein is stained with silver and the arrow indicates the dye front of the gel.

Figure 29



An autoradiograph of the dried polyacrylamide gel in Figure 28.
Both Peptide I and II are labeled with [^{125}I]-TID.
The arrow indicates the dye front the gel.
Free [^{125}I]-TID is in some of the lanes below the dye front

molecular weights of I and II were estimated on SDS-PAGE at about 8000 Da. The sequence of Peptide I was determined to be the N-terminus of the protein, however the number and position of the radiolabeled residues were not determined. No data is available on the sequence of Peptide II at this time. These peptides may represent the same sequence with different elution patterns due to differential labeling or labeling at alternative residues as observed by Spiess et al. (11) for the sucrase/isomaltase complex. These possibilities could not be assessed due to the presence of free [^{125}I]-TID which prohibited the determination of the degree of labeling and the lack of information on the position of the label on these peptides.

Evidence for an "Active Core":

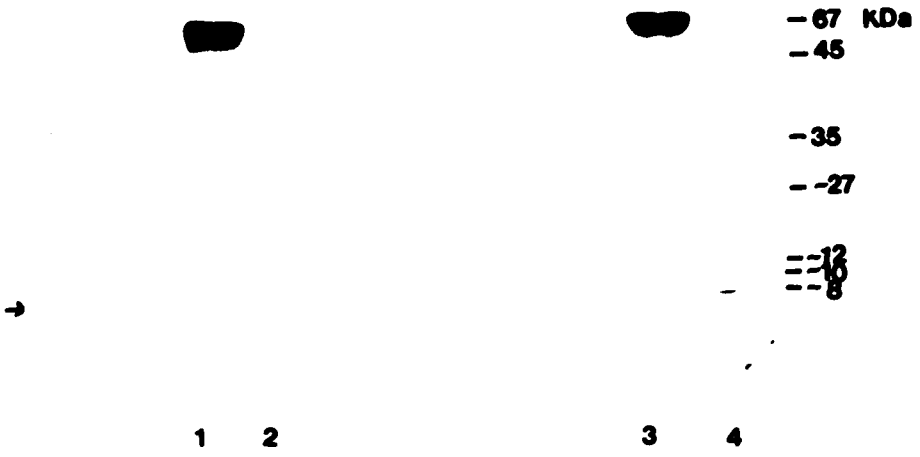
The loss of the β-Glc protein band on SDS-PAGE (Mr = 67,000) demonstrated cleavage by Pronase®, a non-specific protease mix. However, 50 - 90% of the β-Glc hydrolytic activity using 4MU-Glc as a substrate was retained after digestion. Pronase® was shown to lack the ability to cleave 4MU-Glc (data not shown). These results suggest that at least one of the β-Glc fragments retains some of the catalytic ability of the active site. Although, the possibility that the Pronase®-cleaved enzyme retains its active tertiary structure prior to SDS denaturation cannot be ruled out. The native enzyme was resistant to cleavage as evidenced by the 5:1 (wt:wt) ratio of Pronase® to enzyme protein required to observe complete loss of the protein band on SDS-PAGE. Attempts to isolate the active fragment(s) were hindered by the presence of the large excess of Pronase® protein and the instability of the active fragments on affinity purification.

In an effort to identify β-Glc specific fragments in the Pronase® digest, [^{3}H]Br-CBE, a mechanism-based active site inhibitor (4), was used to affinity label the β-Glc fragments. An autoradiograph of the [^{3}H]Br-CBE labeled fragments after SDS-PAGE is shown in Figure 30. Pronase® does not bind [^{3}H]Br-CBE. Lanes 1 and 3 are the labeled, uncleaved β-Glc. Lane 4 is a Pronase® digest of the labeled enzyme and Lane 2 shows the peptides which bound [^{3}H]Br-CBE after cleavage (Mr = ∿8, ∿10 and ∿12 kDa). The presence of the inhibitor in the active site may confer some protection as evidenced by the presence of additional higher molecular weight fragments in Lane 4 (Mr = ∿27, 35 and 45 kDa). These results suggest that Pronase® trims the protein proteolytically to an "active core" which may be degraded further retaining the ability to bind the [^{3}H]Br-CBE.

Discussion

Acid β-glucosidase is a tightly membrane associated protein which requires detergents for solubilization (12). Prior to these studies, the structural properties of the membrane associated region have not been investigated. Domains and regions in contact with the membrane are generally assumed to consist of hydrophobic amino acid residues which are relatively inert and require a highly reactive reagent as a label. [^{125}I]-TID, which partitions almost exclusively to the lipid core of the membrane, has been used to study the membrane associated regions and domains of a number of proteins (10, 11, 13, 14) and, in several cases, only the residues exposed to the lipid core of the membrane have been shown to be labeled (13, 14). Using this label,

Figure 30



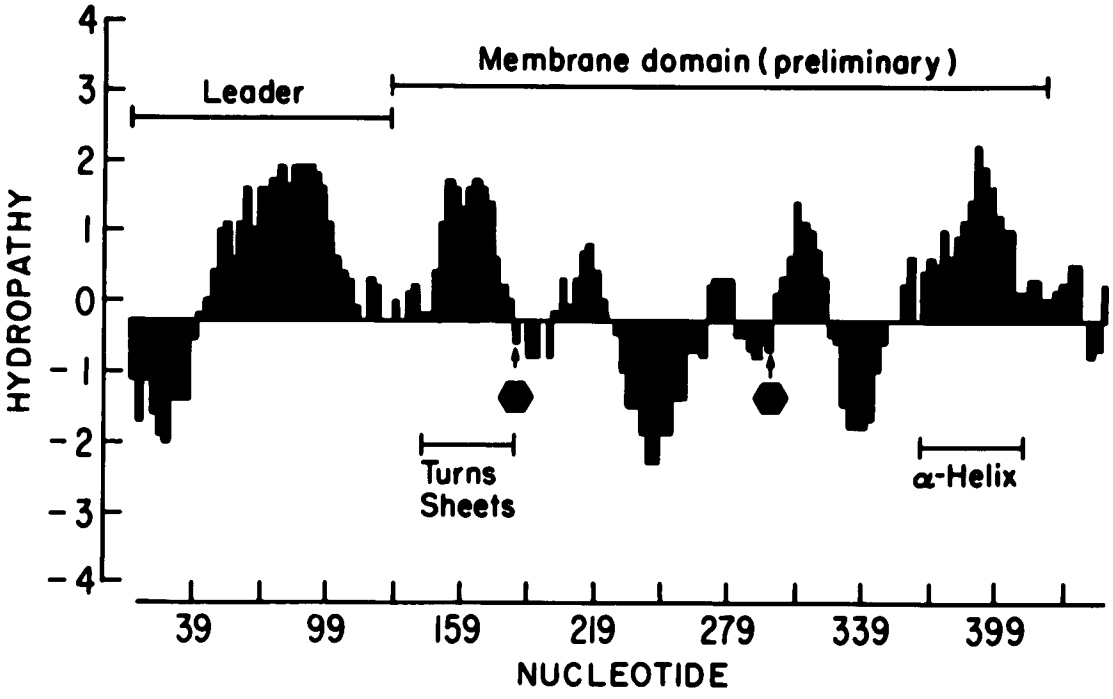
An autoradiograph of the [^{3}H]Br-CBE labeled acid β-glucosidase and the fragments generated by Pronase® cleavage
Lanes 1 and 3 are the non-proteolyzed labeled enzyme.
Lane 2 shows the peptides which bound [^{3}H]Br-CBE after cleavage (Mr = ∿8 - 10 kDa). Lane 4 shows the peptides generated from cleavage of the labeled enzyme (Mr = ∿8 - 45 kDa).
The arrow indicates the dye front of the gel.

a possible membranous segment of β-Glc has been isolated and identified near the N-terminus of the protein.

The calculated hydropathy profile of the N-terminus, shown in Figure 31, indicates two regions of hydrophobicity between nucleotides 147 - 171 and nucleotides 370 - 423 of the cDNA sequence (15) which correspond to amino acids 14 - 21 and amino acids 88 - 105 of the protein, respectively. The predicted α-helical secondary structure and the stretch of 18 amino acids in the latter region suggests it is a strong possibility for the membranous segment. This suggestion would indicate a molecular weight of about 10,000 Da for the isolated peptide rather than the estimated 8000 Da. However, studies have suggested that the intrinsic charge and shape of small peptides are more important for determining mobility on SDS-PAGE, particularly for those of a hydrophobic or amphipathic nature (16). The cluster of charged amino acids near the N-terminus and the possible N-linked glycosylation at Asn^{19} and Asn^{59} (Fig. 31) reveals an amphipathic property of this peptide which supports the inaccuracy of the estimated molecular weight.

The presence of two prolines (amino acid 98 and 99) in the middle of the hydrophobic region at nucleotides 400 - 405 suggests the interruption of the α-helical structure. In conjunction with the fact that 23 amino acid residues in α-helical structure are required to traverse the membrane (11), the amphipathic nature of the segment and the possible interruption of the α-helix are consistent with a proposed structure in which the N-terminal sequence and possible glycosylation site are extramembranous. These results also suggest that the hydrophobic region forms an intramembranous hairpin structure and the

Figure 31



Hydropathy profile of the leader and N-terminal sequences of acid β-glucosidase. The N-terminus has been tentatively identified as a membranous segment of the enzyme. Probable secondary structure is outlined below the profile and the hexagonal symbol represents N-linked glycosylation consensus sequences.

active site domain of the protein (near the carboxy terminus) is in the lysosome. This topology would suggest that cleavage at a site between the membranous domain and the non-membranous portion of the protein would release an active, soluble fragment. The studies using Pronase® provide evidence for the existence of this site and the release of an "active core". The binding of a proteolytically insensitive catalytic domain to a membrane domain through a more sensitive boundary region has been observed for other proteins (17, 18, 19, 20). Investigations of the solubility properties and the N-terminal sequence of the isolated active fragment after controlled proteolysis, similar to studies of mannosidase II (20) and the sucrase/isomaltase complex (11), and the determination of the labeled residues should provide further critical information on the topology and domain structure and function of β-Glc.

Recent work by Barriocanal et al. (21) using monoclonal antibodies indicated that the transport of lysosomal membrane proteins are independent of N-linked glycosylation pathways used by soluble lysosomal proteins (22). Further support is derived from the fact that β-Glc is not absent in the lysosomes from patients with I-cell disease (23), a disorder of the mannose-6-phosphate receptor pathway of lysosomal protein transport (24). Barriocanal et al. (21) suggested that sequences within the membranous domain of lysosomal membrane proteins serve as a transport signal. The mechanism by which this domain in β-Glc facilitates lysosomal localization and its relationship to the other β-Glc domains are subjects of further investigations at a molecular level.

Conclusion

Studies of the residual activity in cells from patients with the subtypes and variants of Gaucher disease delineated two major classes of mutant β-Glcs: 1) those which had normal interactions with amphiphiles and inhibitors; and 2) those which had abnormal interactions with these effectors of β-Glc activity (1). The former class included most non-Jewish Type 1 and Types 2 and 3 Gaucher disease. The latter included the Ashkenazi Jewish Type 1 variant as well as some non-Jewish Type 1 Gaucher disease patients. The research presented in this thesis was directed to elucidating the basis of the abnormalities of β-Glc function in the AJGD Type 1 enzyme. The working hypothesis of the causal mutation(s) in this variant of Gaucher disease was a single base substitution leading to an amino acid alteration in or near the active site.

Initial studies were directed to determining the structure and function of the normal β-Glc as a framework for understanding the functional defect of the AJGD Type 1 enzyme. Based on previous inhibitor and modifier studies of the normal enzyme, a three domain model of the active site was proposed: 1) the catalytic site which has specificity for glycon head groups; 2) the aglycon binding site which interacts with the alkyl and acyl chains of some substrates and inhibitors; and 3) the "third" domain which binds sphingosyl moieties as well as the negatively-charged lipids, taurocholate and phosphatidylserine (2). The objective of the studies of the normal enzyme was to determine the structural equivalents on the enzyme for each of the proposed domains and to elucidate their functional properties.

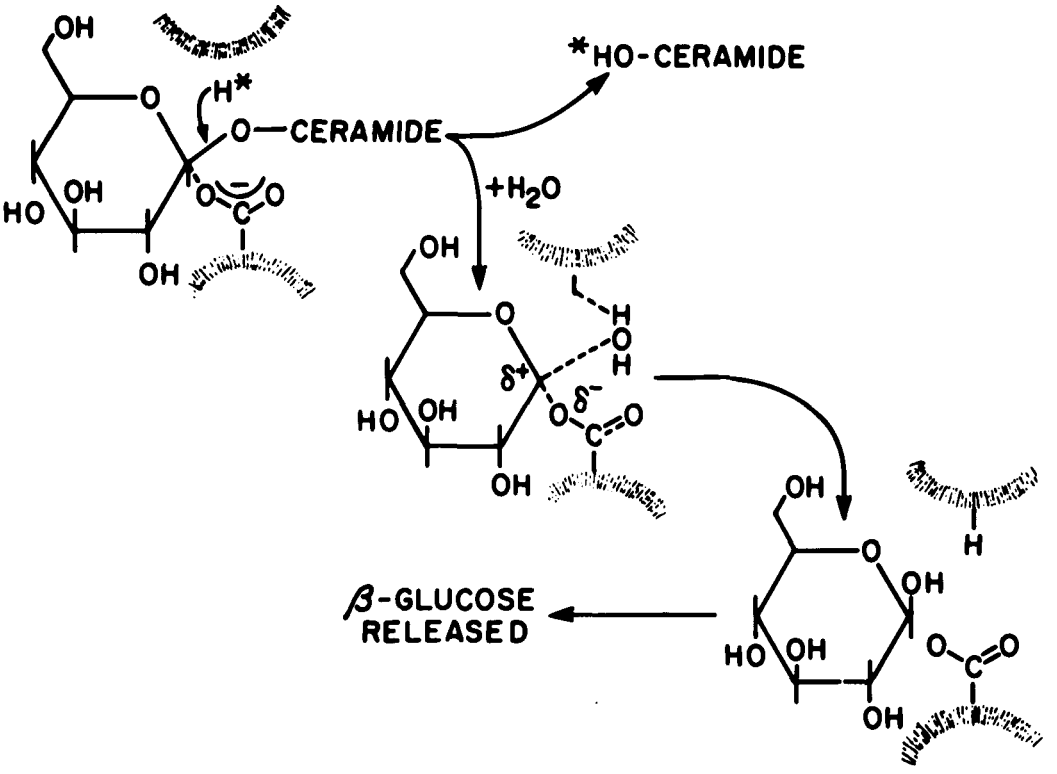
Studies of the pH modulation of substrate hydrolysis as well as the covalent inactivation by CBE identified important amino acid residues in the active site and provided insight into the mecchanism of action β-Glc. Substrate hydrolysis (4MU-Glc) was dependant on the proper protonation of two amino acids in the active site with pKa values of 4.6 and 6.7. Asp^{443} was identified by amino acid sequencing of peptides from [^{3}H]-Br-CBE affinity labeled homogeneous placental β-Glc as the nucleophile corresponding to the pKa=4.6 group. These studies located a portion of the single active site near the carboxy terminus of the β-Glc protein subunit and represented the first such assignment for a human lysosomal hydrolase. The proton donor for hydrolyis has been identified only by its pKa=6.7 and the solvent shielded environment of the active site hinders assignment of this residue to a particular amino acid. Recent studies (Legler and Grabowski, unpublished observations) with the bovine β-Glc suggest that the pKa=6.7 group is a neutral acid. Consequently, the active site of β-Glc may contain two carboxylic acid residues for the proton donation and nucleophilic attack required for substrate hydrolysis. Although the location of other critical residues have not been determined, the fact that fragments of β-Glc(Mr=10,000-45,000) retain the ability to bind CBE and hydrolytic activity is retained by some of these fragments indicates that the entire functional active site can be isolated. Once the active fragments are purified, the integrity of the function of the proposed aglycon binding and "third" domains and their structural equivalents can be determined.

The inhibitor binding studies indicated that the hydrolytic reac-tion proceeds through a binary complex which contains a single

proton and that the kinetic mechanism is ordered uni-bi. The source of the proton in the binary complex of the inhibitor and enzyme was ambiguous, since most of the potent inhibitors were amines or imines which had pKa values similar to the pKa=6.7 group on the enzyme. However, the studies with nojirimcin (pKa=5.18) suggested that the proton is donated from the enzyme. Irrespective of the source of the proton, the linearity of the slope and intercept replots of the potent nitrogen-containing inhibitors and the competitive inhibition by a β-glucose indicate that dNM derivatives and nojirimycin resemble the last product released from the enzyme (i.e., β-glucose). The linear non-competitive inhibition of the enzyme by GS derivatives indicated that these compounds more closely resemble the first product released (i.e., ceramide or 4MU). The overall reaction mechanism is summarized in Figure 32 and indicates that ceramide is released first and β-glucose is the second product.

The mechanism of hydrolysis by β-Glc and the proposed active site model are similar to those reported for other glycosidases and complex lipid binding proteins. Based on the classification of glycosidases proposed by LaLegerie et al. (3), human β-Glc is a class 2 glycohydrolase which is similar to almond β-glucosidase (4) in its interaction with unprotonated inhibitors and its hydrolytic mechanism. Using the almond β-glucosidase as a model, further studies of the catalytic mechanism of human β-Glc may reveal properties of the transition state. This finding may prove to be important to the understanding of the AJGD Type 1 mutation, since the k_{cat} value for this defective enzyme was normal as determined by aglycon release (see Table 6). This finding led to the conclusion that the 5-10 fold abnormal binding of particular inhibitors at the active site was unrelated to the hydrolysis of

Figure 32



A schematic representation of the proposed uni-bi mechanism of glucosyl ceramide hydrolysis by acid β-glucosidase. The figure indicates the donation of a proton to the bound substrate followed by release of ceramide, the first product. The addition of water releases the second product, β-glucose.

substrates. Although this was supported by the normal binding of nojirimycin and castanospermine, a unified hypothesis of the AJGD Type 1 mutation would include abnormal hydrolysis related to to the residues involved in binding of these inhibitors. Future studies should clarify this difficulty.

The possibility that all complex lipid binding proteins share a similar domain structure to that proposed for β-Glc is of particular interest. Recent physical biochemical studies of the phosphatidylcholine transfer protein (PC-TP) (5)and the sphingolipid activator protein one (SAP1)(6) indicate the presence of three domains for specific interaction with components of the respective lipids. For PC-TP, the fatty acid acyl chains were bound at a 60-90° angle to each other and the hydrophilic domain had specifity for the polar head group of the lipid. Using radioactive carbene analogues (7), Tyr^{54} was covalently labeled and shown to be shared by both fatty acid acyl binding domains. The results of these structural studies suggests that the binding of the fatty acid acyl and alkyl chains of ceramide to the active site of β-Glc may have a structural basis in residues which are shared by the aglycon and "third" domains.

More striking structural similarity was evident between the proposed active site of β-Glc and the binding domains of SAP1. The model of the sphingolipid binding domains of SAP1 includes not only a glycon binding domain and a hydrophobic domain but a shared region which contains residues with specifity for the amide and hydroxyl groups of the sphingolipids. This is precisely the model that was proposed (see Figure 26) for the β-Glc active site based on kinetic data with alkylglycons. Such similarities in the binding domains for these complex

lipid binding proteins suggests a common evolutionary ancestor prior to the advent of specific enzymatic activities for the sphingolipid hydrolases. The fact that β-Glc does not require a specific activator protein for the binding of the lipoidal substrate suggests that it mayhave an alternative evolutionary pathway from the soluble lysosomal hydrolases which appear to require such cofactors to complete their active sites (8).

In addition to the active site, β-Glc requires a region on the enzyme for membrane association. The preliminary data presented in this thesis indicate that a domain near the N-terminus of the protein serves this function. The location and predicted secondary structure of this sequence and the presence of a glycosylation concensus sequence at Asn^{19} (see Figure 30) suggest that a portion of β-Glc is inserted into the membrane: a domain near, but not including, the N-terminus is tightly associated with the lysosomal membrane and the active site is in the interior of the lysosome. This location and orientation could provide an explanation for the lack of a required activator protein for presentation of the substrate to the active site, since the lysosomal membrane could provide this function.

The research presented in this thesis serves as a framework for further structural and functional studies of the normal and Gaucher disease enzymes. From these and other investigations, the mutations which are causal to the subtypes and variants of Gaucher disease have their major effects on the intracellular stability of β-Glc. These destabilizing mutations likely lead to increased susceptibility to proteolytic digestion with resultant decreases in β-Glc activity in the lysosome.

The elucidation of these mutations would provide further insight into the basis of the various forms of Gaucher disease as well as a better understanding of the biology of this membrane associated lysosomal hydrolase.

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Conclusion

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