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BIOCHEMICAL AND IMMUNOLOGIC STUDIES OF ACID ALPHA-GLUCOSIDASE DEFICIENCY, A GENETICALLY HETEROGENEOUS, INHERITED NEUROMUSCULAR DISEASE

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by

GUNDULA ULLRICH LaBADIE

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

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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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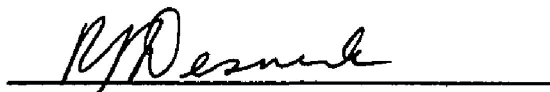

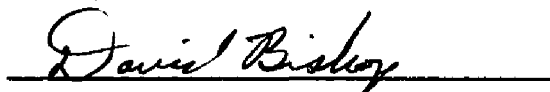

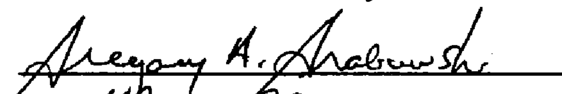

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Abstract

BIOCHEMICAL AND IMMUNOLOGIC STUDIES OF ACID α-GLUCOSIDASE DEFICIENCY, A GENETICALLY HETEROGENEOUS, INHERITED NEUROMUSCULAR DISEASE

by

GUNDULA ULLRICH LaBADIE

Thesis Advisor: Kurt Hirschhorn, M.D.

The overall objectives of these studies were to characterize the normal and mutant acid α-glucosidase (GAA) enzymes using biochemical, kinetic and immunologic procedures, and to characterize the nature of the enzymatic defect(s) in the subtypes and variants of GAA deficiency.

To accomplish these goals, methodologies for the accurate identification (isoelectric focusing), purification (chromatofocusing, electroelution, reversed phase HPLC) and characterization (rocket immunoelectrophoresis, immunoblotting, protein digestion, peptide mapping) of the normal and residual mutant enzyme forms were developed. Further accomplishments include the development of a panel of monoclonal antibodies (Mc Abs) to placental GAA and the identification of a covalent, active site-directed inhibitor (CBE) of the human enzyme.

Rocket immunoelectrophoresis with polyclonal (PC) anti-placental GAA Abs demonstrated extensive heterogeneity among and within the subtypes of glycogenosis II. The residual, catalytically inactive enzyme in the CRM-positive infantile subtype was unique in that it had a reduced immunoreactivity and an abnormal pI. Further immunologic studies using MC Abs showed that MC Ab Sp2/53 recognized three enzyme forms in several infantile and adult subtypes previously classified as

CRM-negative using PC Abs. GAA isozymes 1 and 2, purified by conventional chromatographic procedures, were resolved into four distinct, catalytically active electrophoretic forms by isoelectric focusing. The isolated enzyme forms had the same physical and kinetic properties as the conventionally purified isozymes. The subunit composition changed from predominantly 73 kDa to predominantly 67 kDa with increasing electronegativity. Isozyme 4, the most electropositive of the GAA isozymes, was immunologically, physically and kinetically identical to GAA isozyme 1. These enzyme forms contained no sialic acid residues, and little or no phosphate, indicating that the observed charge heterogeneity was most likely due to differences in the protein backbone of the enzyme.

Tryptic peptide maps of the major protein species identified in purified GAA preparations have been generated. Comparative studies clearly showed the relatedness of the electroeluted 96 (precursor), 73 and 67 kDa (mature) denatured enzyme forms. A 20 kDa form, a glycoprotein which consistently co-purified with GAA, was shown to be unrelated. Finally, the amino acid composition and N-terminal amino acid sequence of the 67 and 73 kDa subunits of GAA isozyme 1 have been obtained. The composition of the subunits was virtually identical. The unblocked, mature 73 kDa enzyme provided an amino-terminal sequence of 16 residues, 50% of which were coded for by low redundancy codons. The amino-terminal amino acid sequence of the 67 kDa subunit was distinct from that of the 73 kDa form.

FOREWORD

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

LaBadie, G.U., Beratis, N.G., and Hirschhorn, K.: Molecular pathology of acid α-glucosidase deficient variants. Pediatr. Res. 16:193A, 1982.

Beratis, N.G., LaBadie, G.U., and Hirschhorn, K.: Genetic heterogeneity in acid α-glucosidase deficiency. Am. J. Hum. Genet. 35:21-33, 1983.

Slonim, A.E., Coleman, R.A., McElligot, M.A., Najjar, J., Hirschhorn, K., LaBadie, G.U., Mrak, R., Evans, O.B., Shipp, E., and Presson, R.: Improvement of muscle function in acid maltase deficiency by high-protein therapy. Neurology 33:34-38, 1983.

Beratis, N.G., LaBadie, G.U., and Hirschhorn, K.: Acid α-glucosidase: Kinetic and immunologic properties of enzyme variants in health and disease. In: Isozymes: Current Topics in Biological and Medical Research, Vol. II, Rattazzi, M.C., Scandalios, J.G., and Whitt, J.G., eds., Alan R. Liss, Inc., New York, pp. 25-36, 1983.

LaBadie, G.U., Harris, H., Beratis, N.G. and Hirschhorn, K.: Monoclonal antibodies to acid α-glucosidase: Further evidence for genetic heterogeneity in Pompe disease. Am. J. Hum. Genet. 37:A12, 1985.

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LaBadie, G.U., Beratis, N.G., and Hirschhorn, K.: Biochemical and immunologic characterization of acid α-glucosidase deficient variants. Am. J. Hum. Genet., in review.

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LIST OF ABBREVIATIONS

Antibody	Ab
Bovine serum albumin	BSA
Conduritol B epoxide	CBE
Cross-reacting material	CRM
Castanospermine	CS
Deoxynojirimycin	dNM
Ethylenediaminetetraacetic acid	EDTA
Enzyme-linked immunosorbent assay	ELISA
Fetal calf serum	FCS
Acid α-glucosidase (EC 3.2.1.20)	GAA
Isoelectric focusing	IEF
Monoclonal	MC
4-Methylumbelliferyl-α-D-glucopyranoside	4MU α-Glc
N-Dodecyldeoxynojirimycin	N-C_{12}-dNM
Nojirimycin	NM
Polyacrylamide gel electrophoresis	PAGE
Phosphate buffered saline	PBS
Polyclonal	PC
Sodium dodecylsulfate	SDS

I. OBJECTIVES

GAA deficiency (Pompe disease; glycogenosis II) is an autosomal, recessive disease characterized by the pathologic intralysosomal accumulation of glycogen, primarily in muscle. Three subtypes of glycogenosis II have been identified, the infantile, juvenile and adult subtypes. Although the primary metabolic defect in each of these is the deficient activity of GAA, there is remarkable heterogeneity in the clinical manifestations of these disorders. Four isozymes of GAA, each encoded by a different allele at the same locus, have been recognized. However, the interrelationship of the allelic isozymes at the GAA locus and their relative contributions to the clinical manifestations in the various subtypes of glycogenosis II have not been examined.

The overall objective of this research was to investigate the nature of the observed clinical and biochemical heterogeneity. The specific aims of this research included:

1. Screening of placentae and cultured skin fibroblasts from normal individuals and patients with glycogenosis II to determine the pattern and frequency of the four isozymes (types 1, 2, 3 and 4) of GAA.

2. Purification of the GAA isozymes, and of the electrophoretic forms unique to each of three common isozymes (types 1, 2 and 4) for comparative physical, kinetic and immunologic studies.

3. Production of a panel of anti-GAA MC Abs to investigate the structure and function of the electrophoretic forms of the enzyme.

4. Investigation of the mechanism of action of the various forms of GAA using a variety of substrate analogues, both active site-directed

inhibitors and others, in the presence of natural and artificial substrates.

5. Determination of the epitope composition of native, denatured enzyme forms and enzymatically generated peptides of normal and mutant GAA using PC and MC Abs.

6. Determination of the N-terminal (and internal peptide) amino acid sequence of the precursor and mature forms of GAA in order to synthesize oligonucleotide probes and demonstrate colinearity with cDNAs encoding GAA.

II. BACKGROUND AND RATIONALE

A. Clinical Heterogeneity in GAA Deficiency:

Glycogenosis type II is an autosomal recessive disease resulting from the deficient activity of lysosomal GAA (EC. 3.2.1.20). The enzyme defect leads to the pathologic accumulation of glycogen, primarily in muscle (1). The enzyme has α-1,4 and α-1,6 glucosidase activity using maltose and isomaltose as substrates, and catalyzes the total conversion of glycogen to glucose (2). The enzyme also hydrolyzes the synthetic substrates, 4-methylumbelliferyl-α-D-glucoside (4MU α-Glc), β-napthyl-α-D-glucoside, and 6-bromonapthyl-α-D-glucoside. The binding of GAA to Sephadex, a substrate analogue glucose polymer containing predominantly α-1,6 linkages, has been documented (3).

Most notably, GAA deficiency is clinically heterogeneous; the infantile, juvenile and adult subtypes are distinct clinical entities (4,5). In the infantile or generalized type (Pompe disease), enzymatic activity is absent in all tissues. Onset of symptoms occurs within the first weeks of life, and glycogen accumulation is massive, especially in muscle, heart, and the central nervous system. Patients with this subtype die from cardiorespiratory failure within the first two years of life (5,6). Onset of symptoms in the juvenile type occurs during the first or second year of life. Progressive muscular weakness is the major symptom, cardiac or visceral involvement is rarely evidenced, and patients die during the first or second decade (7,8). In the adult-onset, or muscular type, the onset of symptoms occurs in the third or fourth decades. The main clinical manifestation is slowly progressive proximal limb weakness (9,10). Glycogen content in muscle is only

slightly increased or normal. Cardiomegaly is absent, and death from respiratory failure occurs many years after the onset of symptoms. Both the juvenile and the adult subtypes have residual enzyme activity (5-30% of normal) (9-11). Furthermore, the age at onset, the spectrum of clinical manifestations and disease severity in the juvenile and adult-onset subtypes are highly variable among families, providing evidence for the occurrence of genetic heterogeneity within these subtypes (12). For example, two patients with the juvenile subtype had liver glycogen levels which were nearly normal but had no GAA activity in either muscle or liver. A third patient lacked GAA activity in muscle, but had normal levels of activity in the liver (7). Hlinak et al. (13) reported a variant with glycogen storage in skeletal muscle, decreased GAA activity in muscle and liver, and normal activity in leukocytes. Another juvenile patient had marked glycogen accumulation in muscle, slightly decreased enzymatic activity in leukocytes, and complete absence of GAA activity in fibroblasts (13). Most patients with the adult subtype demonstrate a residual GAA activity of 5-20% of normal (14-16). However, patients demonstrating muscle glycogen accumulation in the presence of normal or elevated levels of GAA activity in urine and muscle also have been described (17). The clinical course of these variants with no demonstrable enzyme defect remains unknown.

B. GAA Isozymes:

GAA has been shown to be polymorphic (18). The polymorphism is detectable using starch gel affinity electrophoresis, but not with agarose or cellogel electrophoresis. The common isozyme, designated type

1, binds tightly to the starch support and migrates slowly toward the anode. About 1 in 16 Europeans is heterozygous and has a pattern with two isozymes, the slow band plus an additional component, designated type 2, which migrates faster toward the anode, indicating a reduced affinity for the starch medium. Isozymes 1 and 2 are determined by two alleles at the GAA locus on chromosome 17 with frequencies of 0.97 and 0.03, respectively (18). A third allele (type 3), which is apparently rare, occurs in Malaysians of Indian ancestry (19) and is detectable using starch affinity electrophoresis. More recently, Nickel and McAlpine (20) identified a fourth allele at the GAA locus, designated type 4, which is detectable only by isoelectric focusing (IEF). This allele appears to be more common than the type 2 allele, occurring at a gene frequency of 0.06. No biochemical or immunologic studies have been performed to characterize the properties of isozyme 4.

C. Biosynthesis and Maturation of GAA:

Lysosomal enzymes are initially synthesized as precursors having higher molecular weights than the mature forms found in lysosomes. Both proteolytic and glycolytic processing of the precursors have been reported (21). Mutations in genes coding for lysosomal proteins or for their processing enzymes may affect lysosomal enzyme activities by: 1) a decreased rate of synthesis; 2) synthesis of catalytically inactive mutant enzyme (including abnormalities in transport and stability); 3) failure to transport enzymes into lysosomes; 4) an increased rate of degradation, and 5) a decreased concentration of an activating or stabilizing factor in lysosomes.

GAA activity is reduced by approximately 80-90% in fibroblasts from patients with the adult subtype (16). Since no kinetic, physical or immunological parameter of the adult mutant GAA was found to be abnormal, it was hypothesized that the enzyme was either synthesized at a reduced rate or degraded at an increased rate (16). Reuser and Kroos (15) studied the secretion, uptake and stability of the normal fibroblast enzyme and the mutant residual enzyme in fibroblasts from the adult subtype. Results of their studies showed that both the precursor and the mature forms of GAA from adult subtype fibroblasts had normal stabilities and normal specific activities. Additionally, the apparent half-lives of the mutant and normal enzymes were comparable. However, NH_4Cl-stimulated secretion of the precursor form into the culture media was markedly reduced in the adult subtype cells. These results suggested a defect in enzyme synthesis which led to a quantitative deficiency of qualitatively normal enzyme.

Similar results were obtained by Steckel et al. (22), using immunoprecipitation of radiolabeled enzyme, gel electrophoresis and fluorography. They found that the rate of NH_4Cl-induced secretion of radiolabeled precursor was less than 15% of that in control fibroblasts. Furthermore, the amount of radioactivity recovered in mature (processed) GAA after long incubations also was less than 15% of the controls. No incorporation of radioactivity into GAA (precursor or mature form) was detected in fibroblasts from patients with the infantile subtype. These authors also used short pulse-labeling to demonstrate that the lower level of the steady state activity of the enzyme in adult subtype fibroblasts from one patient resulted from an enhanced rate of degrada-

tion which occurred prior to transfer into the lysosomes. Although the precursor form of GAA was synthesized at 60% of the normal rate in adult subtype fibroblasts, it was degraded almost immediately.

The development of MC Abs to GAA (23) has provided a further tool to study the synthesis and processing of this lysosomal enzyme. Using a MC Ab that distinguishes between the precursor of GAA, and the intermediate and mature forms of the enzyme (24), Tager and his co-workers (25) were able to define several of the steps involved in the synthesis and processing of GAA. The first identifiable precursor of GAA had a M_r of about 110,000 and was phosphorylated. A proteinase converted the precursor to an intermediate form of M_r 95,000. The intermediate form was subsequently converted to a mature form of M_r 76,000 and 70,000. Their results further suggested that a disulfide bridge was formed during processing of the normal enzyme (25). However, very little is known about the proteolytic maturation of lysosomal enzymes, and more studies on a wide range of mutational types are necessary to determine the underlying cause of the enzymatic deficiency in the various subtypes of glycogenosis II. These proposed studies will identify mutations resulting in abnormal enzyme protein structure and function. These mutations will then be examined at the molecular level using cloned GAA cDNA to determine the precise nature and heterogeneity of their molecular lesions.

D. Kinetic Characterization of GAA:

Studies by a number of investigators, using a variety of species and tissue sources, have demonstrated that lysosomal GAA has very complex kinetic behavior.

Jeffrey et al. (26), in a study on purified GAA from rat liver lysosomes using oligosaccharide and polysaccharide substrates, obtained data consistent with the hypothesis that the enzyme had multiple catalytically active binding sites in close proximity to each other, and which, therefore, interact with each other. One catalytic site bound the disaccharide, maltose, as well as other low molecular weight maltosidically linked oligosaccharides. This same site also had an affinity for isomaltose, an α-1,6-glucosidically linked disaccharide. A second catalytically active site(s) bound polysaccharide substrates such as glycogen. In addition to these two substrate binding site(s), the authors also presented evidence for the existence of an inhibitory site on the enzyme at which maltose and other low molecular weight oligosaccharides of glucose with α-1,4-glycosidic bonds can be bound. Neither polysaccharides, such as glycogen, nor isomaltose had an affinity for this site.

Very similar conclusions were drawn by Palmer (27,28) using GAA from rabbit muscle. His data supported the existence of at least two specific substrate binding sites or subsites, one for the binding of maltose and other oligosaccharides, another for binding polysaccharides such as glycogen. The site appeared to be in close proximity and interacted directly in transglucosylation reactions. Further support for multiple catalytically active binding sites can be found in the work of Rosenfeld

and Belenki [(29) rabbit liver GAA], Fujimori et al. [(30) bovine spleen GAA], and Koster and Slee [(31) human liver GAA].

Although all of these authors concur on the existence of multiple active sites, their studies also demonstrate a great many inconsistencies in pH optima, cation requirements, the type and extent of inhibition by various inhibitors and substrate analogues, substrate specificities, molecular weight and subunit composition (26-31) Some of these inconsistencies may have been due to the different enzyme sources used, the degree of purity of the preparations, as well as experimental design.

In an effort to explore both the active site topology and mechanism of action of the enzyme, investigators have used a variety of reversible and active site-directed inhibitors. Although most of the inhibitors, which included simple sugars and sugar derivatives, amines and phenols, were extensively applied to studies of the β-glucosidases (32), a number also have been found to be potent inhibitors of GAAs. Two such compounds are nojirimycin, an antibiotic produced by several strains of _Streptomyces_, which differs from D-glucose only by the substitution of an N-group for oxygen in the pyranose ring (33), and the active site-directed inhibitor, conduritol B epoxide (CBE; 3,5/4,6-tetrahydroxycyclohex-1-ene), first introduced by Legler (34,35). Nojirimycin was found to be three orders of magnitude more potent an inhibitor of purified human liver lysosomal GAA than the classical disaccharide inhibitor, turanose. Unlike turanose, which is a competitive inhibitor, the hydrolysis of maltose, glycogen and 4MU α-Glc was inhibited by nojirimycin in a noncompetitive and an uncompetitive fashion, respectively. Furthermore, nojirimycin had little or no effect on the hydro-

lysis of substrates other than α-D-glucose derivatives. Thus, although nojirimycin is a relatively specific inhibitor of GAA activity, the compound exerts its influence not at the active site, but on residues surrounding the site, which are essential for optimal activity of the enzyme.

Direct analysis of active site centers is possible using active site-directed inhibitors such as CBE (34). This compound was found to covalently inhibit the sucrase-isomaltase complex from rabbit small intestine (36), yeast β-fructosidase (37) and GAA from *Monascus ruber* (34). Using a number of protein side chain modification reagents, the latter study suggested that a carboxylate(s) of aspartate or glutamate was the reactive nucleophile(s) with CBE, and that this group is essential for GAA activity. No studies using this suicide inhibitor with a human enzyme source have been reported to date.

E. Biochemical and Immunologic Studies of the Genetic Heterogeneity in GAA Deficiency:

1. *Purification and Characterization of Human GAA*: Studies conducted in the laboratory of Drs. K. Hirschhorn and N.G. Beratis, on which the current thesis work is based, were aimed at correlating the observed clinical heterogeneity in GAA deficiency with specific defects (structural and/or functional) in the residual enzyme proteins. For this purpose GAA was purified to homogeneity from human placentae, normal cultured skin fibroblasts and cultured fibroblasts from patients with the adult and juvenile subtypes. The catalytic properties of these enzymes were determined, and the placental enzyme was used for the

production of PC Abs (38) and MC Abs. The physicokinetic properties of the purified normal and residual enzymes including K_m, thermostability, pH optima and pI, were identical. Additionally, the purified enzymes were immunologically identical when tested against rabbit anti-placental GAA Abs by agar double immunodiffusion and immunoprecipitation (38). The polyclonal monospecific rabbit Abs were subsequently used to develop a sensitive, quantitative rocket immunoelectrophoretic system which demonstrated structural and quantitative differences between the normal and the mutant enzyme proteins (38-41).

Additionally, isozyme 2, which was shown to have a reduced affinity for the starch matrix in an affinity electrophoretic system (18), was purified to near homogeneity from a heterozygous (isozymes 1-2) placenta using conventional separation techniques (42). Isozyme 2 eluted from a DEAE-cellulose column at a lower salt concentration than isozyme 1, indicating that it had a greater positive charge. The pH optimum, thermal stability, K_m and V_{max}, using maltose as substrate, were identical for the two isozymes. However, isozyme 2 demonstrated atypical kinetics and a greatly reduced catalytic activity toward glycogen (8 to 20% of the activity of isozyme 1 at non-saturating and saturating substrate concentrations, respectively). That this reduced activity was not due to the inability of isozyme 2 to cleave the α-1,6 linkages in glycogen was demonstrated using isomaltose (6-O-α-D-glucopyranosyl-D-glucose) as substrate (42). The reduced catalytic activity toward glycogen also was documented in crude preparations of heterozygous [1-2] placentae and fibroblasts (43,44). No immunologic differences were detected between isozymes 1 and 2 using PC Abs (42). These studies indicated that isozyme 2 may be of clinical significance and it

was hypothesized that individuals homozygous for this isozyme may develop muscular dystrophy-like symptoms late in life, similar to patients with the adult subtype of glycogenosis II.

2. Characterization of the Biochemical Heterogeneity in the GAA Deficient Subtypes: Using a variety of biochemical, electrophoretic and immunologic techniques, the presence of extensive biochemical heterogeneity was demonstrated among and within the three major subtypes of glycogenosis II. Furthermore, these studies indicated that the various subtypes of glycogenosis II resulted from a family of mutations which alter GAA. The mutations identified by our laboratory which result in GAA deficiency are listed below.

a) Absence of the enzyme protein, or the presence of an enzyme protein which is so altered that it has lost both catalytic and immunologic properties (CRM-negative infantile and adult subtypes) (38-41). Ten of 11 patients examined with the infantile subtype (GM-248; GM-244; GM-4912; GM-3329; WG-285; WG-482; WG-173; B-4; M-2478; C-482) demonstrated complete absence of GAA protein. One of three patients with the adult subtype studied was found to be CRM-negative (GM-1935); complete absence of enzyme activity was also demonstrated in the muscle of this patient. It is still not clear why this patient developed the adult subtype, rather than the infantile subtype of the disease.

b) Presence of an enzyme protein with severely reduced catalytic activity (CRM-positive infantile subtype) (39-41). Only one (M-4581) of 11 patients with infantile GAA deficiency had a CRM-positive mutation. The catalytically inactive protein in fibroblasts was quantitated using

rocket immunoelectrophoresis and was shown to be reduced by 50-70% of normal levels (39).

c) Reduction in the amount of enzyme protein with a proportional reduction in enzyme activity (adult subtype) (38-41). Two of the three patients with the adult subtype (M-3773; GM-443) had a residual enzyme activity of 9-22% of normal; a similar amount of residual enzyme protein was detected by quantitative immunoelectrophoresis. The residual fibroblast enzyme protein from one of these patients (M-3773) was purified to homogeneity and was shown to have normal physicochemical and kinetic properties (38).

d) Severe enzyme deficiency in muscle with moderate reduction of the activity in fibroblasts (juvenile subtype) (39,40,45). Fibroblasts from this patient (J.N.) had an enzyme activity of 16-28% of normal, values consistently higher than those observed in fibroblasts from patients with the adult subtype. This reduction in enzyme activity was directly proportional to the amount of enzyme protein detected by quantitative rocket immunoelectrophoresis (39,40). Importantly, no enzyme activity or protein was detected in a muscle biopsy of this patient, which also showed increased glycogen accumulation (45).

e) Presence of an isozyme with both reduced catalytic activity for glycogen and a reduction in the number of enzyme molecules in fibroblasts (cultured fibroblasts with the genotype 2-2 of GAA) (40-42). Whether or not subjects homozygous for isozyme 2 of GAA will develop muscular weakness later in life, similar to that observed in the adult subtype of the disease, remains unknown at the present time and is under investigation.

f) Massive intralysosomal glycogen accumulation in muscle in the presence of normal to elevated levels of GAA activity and protein (17). The enzyme protein in crude fibroblast lysates had normal physical, kinetic and immunologic properties. The defect in this patient (A.E.) may, therefore, represent a mutation in the processing or compartmentalization of the enzyme.

III. MATERIALS

The following were from commercial sources: Maltose, isomaltose, trehalose, turanose, α-methylglucoside, 1-thio-β-D-glucose, 5-thio-D-glucose, α- and β-glucose-1-phosphate, L-glucose, D-glucose-L-cysteine, N-acetylglucosamine, glucosamine-1-phosphate, 4-methylumbelliferyl-α-D-glucoside (4MU α-Glc), acid and alkaline phosphatase, Nonidet P-40 and Triton X-100 (Sigma Chemical Co., St. Louis, MO); castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine), mouse immunoglobulin subtype identification kit and glycogen (Boehringer Mannheim, Indiannapolis, IN); D-glucosamine hydrochloride (Aldrich Chemical Co., Milwaukee, WI); sodium dodecyl sulfate (SDS; British Drug House, Poole, UK); hydrolyzed starch (Connaught Laboratories Limited, Willowdale, Ontario, Canada); agarose type LSL, _S. aureus_ V8 protease and bovine serum albumin (BSA) (Miles Scientific, Naperville, IL); fluorescein-labeled Concanavalin A (Calbiochem-Behring Corp., LaJolla, CA); materials for polyacrylamide gel electrophoresis (PAGE) and Western blotting (Bio-Rad Laboratories, Richmond, CA); isoelectric focusing (IEF) supplies (LKB Instruments, Inc., Gaithersburg, MD); conventional chromatography and chromatofocusing supplies (Pharmacia Fine Chemicals, Piscataway, NJ); fluorescamine (Pierce Chemical Co., Rockford, IL); TPCK-trypsin (Cooper Biomedical, Malvern, PA); N-glycanase™ (Genzyme Corporation, Boston, MA); vectastain ABC Kit (Vector Laboratories, Inc., Birlingame, CA); nitrocelluose filters (BA85, 0.46 µm; Schleicher and Schuell, Keene, NH); acetonitrile (Burdick and Jackson Laboratories, Meskegon, MI); Vydac 5 µ C4 reversed phase column (4.6 mm x 25 cm; The Separation Groups, Hesperia, CA); nojirimycin (NM), deoxynojirimycin (dNM), N-dodecyl-dNM (N-C_{12}-dNM) and

conduritol B epoxide (CBE; 1,2-epoxy-3,5/4,6tetrahydroxycyclohexane) (generously provided by Dr. Gregory A. Grabowski).

All other reagents were of the highest quality available.

Cultured skin fibroblasts used for these studies were obtained from the Human Genetic Mutant Cell Repository, Camden, NJ and the Repository for Mutant Cell Strains, Montreal, Canada. Additionally, fibroblasts derived from patients diagnosed in the laboratory of Dr. Kurt Hirschhorn were used. The fibroblast strain homozygous for GAA isozyme 2 was obtained from Dr. D.A. Swallow of the Galton Laboratory, University College, London. Normal fibroblast strains used as controls were obtained from subjects tested for heterozygosity of various metabolic disorders and found to be normal.

Placentae, fresh or frozen, were obtained from the delivery room at the Mount Sinai Hospital.

IV. METHODS

A. Tissue Culture:

Skin fibroblasts were cultivated in RPMI-1640 medium supplemented with 12% FCS, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml) (Gibco, Grand Island, NY). Fibroblasts were harvested at early or late (two weeks after passage) confluency by using a short trypsin treatment (46). Harvested cells were washed three times with 0.9% saline and stored at -80°C until used.

B. Purification of GAA:

Isozymes 1, 2 and 4 were partially purified from fresh or frozen placentae using established procedures (42). Briefly, the placental tissue was homogenized in water and centrifuged. The supernatant was batch-eluted with CM-Sephadex C50 to remove hemoglobin and other proteins. The flow-through containing the GAA was precipitated with ammonium sulfate, the precipitate collected by centrifugation, redissolved and dialyzed extansively. The dialysate was concentrated using an Amicon PM-10 membrane and the concentrate was applied to a Sephadex G-100 column. After thorough washing, the GAA bound to the column was eluted with maltose. Isolation of isozyme 4, as well as the multiple electrophoretic forms of isozymes 1 and 2 was accomplished using chromatofocusing. Partially purified isozyme preparations were applied to a 1.6 x 70 cm column packed with polybuffer exchanger PBE™ 94 (Pharmacia) and equilibrated with 0.025 M piperazine-HCl, pH 5.5, at 4°C. The column was developed over a pH gradient of 5 to 4 by eluting with 10 column volumns ($\gtrsim$ 1,200 ml) of 10% (w/v) polybuffer™ 74-HCl, pH 4.0, at a rate of 20 ml/h. Fractions (2 ml) were

monitored for activity with 4MU α-Glc. Peak fractions were individually pooled. The purified enzymes were removed from the polybuffer™ either by ammonium sulfate precipitation or by extensive dialysis against saline.

C. Enzyme Assays:

Determination of GAA activity using maltose, glycogen and 4MU α-Glc have been described in detail (41,42). For highly purified enzyme preparations, 0.8% BSA was included in the incubation mixture to maintain enzyme stability. Under the experimental conditions used, the albumin had no effect on the kinetic parameters studied. For the inhibitor studies, the reaction mix (40 µl) contained buffered substrate and inhibitor and the reaction was initiated by the addition of enzyme. Notable exceptions were studies with CBE. The enzyme was precincubated with CBE for varying periods of time and the reaction was initiated by adding buffered substrate. In most of the experiments, the amount of enzyme was adjusted to insure that less than 5% of the substrate was hydrolyzed.

Protein was measured by the fluorescamine (47) or Lowry (48) method.

D. Preparation of Antisera:

PC Abs to placental GAA were prepared as described previously (38). MC Abs to the enzyme were developed using the hyperimmunization procedure described by Stahli et al. (49). Briefly, a total of 2.2 mg of purified placental enzyme, emulsified in complete Freunds adjuvant (one injection), incomplete Freunds adjuvant (4 injections) or in PBS (2 injections), was injected intraperitoneally into a Balb/c mouse over a period of two months. One day after the last injection, isolated mouse

spleen cells were fused with cell line Sp2/0-Ag14, a non-producing plasmacytoma line, as described (50,51). Hybridomas were selected by growth in HAT media. Cultures were screened for anti-GAA antibodies by ELISA (see below) and the positive cultures were cloned into microtiter plates by limiting dilution in fluid phase.

1. Screening assays for GAA hybridomas: The resulting hybridomas were monitored for Ab production using two different methods: a peroxidase ELISA, which detects both neutralizing and non-neutralizing Abs, and a GAA ELISA, using the synthetic substrate, 4MU α-Glc, which detects only the non-neutralizing Abs.

The peroxidase ELISA screening test was performed as follows. Microtiter wells were coated with rabbit anti-human placental GAA Abs. Unreacted sites were coated with BSA and purified enzyme was added. After thorough washing, hybridoma supernatant was added to the wells, followed by the addition of peroxidase-conjugated rabbit anti-mouse IgG. All wells were washed extensively between each operation. The wells were subsequently assayed for peroxidase.

For the 4MU α-Glc ELISA, rabbit anti-mouse IgG was bound to the microtiter wells. Unreacted sites were blocked with BSA, and the hybridoma supernatant was added. Placental GAA was placed in the wells and allowed to react. After thorough washing, the wells were then assayed for GAA activity using 4MU α-Glc. NaOH was used to stop the reaction and the clones were determined to be positive by detecting the liberated methylumbelliferone under UV light.

2. Ascites Production of Mc Abs: Positive clones were injected intraperitoneally into female Balb/c mice (10-12 weeks) which had been primed with 0.5 ml pristane one week prior to and the day before injection of hybridoma cells (52). The ascitic fluid was collected by paracentesis.

3. Characterization of the Mc Abs to GAA: Immunoglobulin subtypes were determined by the ELISA method using a commercial kit (Boehringer-Mannheim, Indiannapolis, IN).

The isozyme-, subunit- and mutation-specificity of the Mc Abs were determined as follows. Normal and mutant enzymes were subjected to electrophoresis by IEF or SDS-PAGE. The proteins were then electrophoretically transferred to nitrocellulose sheets and stained with the specific Mc Abs. The immunostained proteins were located using an indirect biotin-avidin peroxidase-coupled staining procedure.

For epitope mapping, the enzyme proteins were digested with *S. aureus* V8 protease or TPCK-trypsin (see below). The peptides generated were subjected to SDS-PAGE and processed as described above.

To determine the spacial relationship of the epitopes recognized by the Mc Abs, competition ELISAs were performed as follows: Microtiter plates were coated with purified GAA. The first Mc Ab was applied to the wells and allowed to react for 1 h; the second Mc Ab was then applied and reacted for an additional 1 h. In separate experiments, competition for binding sites on the enzyme molecule also was tested by simultaneously adding both Mc Abs to the coated wells. Finally, peroxidase-conjugated rabbit anti-mouse IgG was added and the peroxidase activity measured. The plates were washed extensively between each step

of the procedure. Under the experimental conditions used, all available Ab-binding sites reacted with each of the respective Mc Abs during the first hour of incubation.

E. Electrophoretic Procedures:

1. SDS-PAGE and electroelution of stained proteins: Samples were electrophoresed by the method of Laemmli et al. (53). For analytical gels, a mini-gel apparatus (Hoeffer, San Francisco, CA) was used at constant voltage (120 V) for 60 min. Electroelution from preparative gels was essentially as described by Hunkapillar et al. (54). A Studier type gel apparatus (55) was used with a 10% gel. Protein was applied to 3 cm wide gel wells and electrophoresed at 50 V for 15 h. After lightly staining the gel with Coomassie Blue R250 (approximately 10 min) and destaining with methanol/acetic acid/water (10:33:157; v/v/v) for 30 min to 1 h, the protein bands were excised with a scalpel, soaked in distilled water for 60 min, and then in elution buffer (0.1% SDS in 0.05 M ammonium bicarbonate) for 10 min. The gel strips were placed in dialysis tubing filled with elution buffer and then were electroeluted at 10 V for 12 to 16 h in a Trans-Blot apparatus (Bio-Rad, Richmond, CA) containing 50 ml of elution buffer. This buffer was replaced with 5-fold diluted elution buffer and elution/dialysis was continued at 20 V for 24 to 48 h. The eluted proteins were concentrated on a Centricon™ 10 Microconcentrator (Amicon Corp., Danvers, MA).

2. Isoelectric focusing: Thin layer, flat-bed isoelectric focusing in the pH range of 4-6 was performed essentially as described. Thin layer IEF acrylamide gels containing 10 ml of 29.1% (w/v) acrylamide, 10 ml 0.9% (w/v) N,N'-methylenebisacrylamide, 36.6 ml double distilled

deionized water with 7.5 g sucrose dissolved in it, 2.5 ml pH 4-6 ampholyte, 0.5 ml pH 3.5-10 ampholyte, and 0.5 ml 0.004% (w/v) riboflavin were poured between two glass plates separated by a thin gasket. After polymerization under fluorescent light, the gels were "aged" for a minimum of 24 h as 4°C. The gel was prefocused for 45 min by increasing the voltage in a step-wise fashion until a voltage of 850 V was reached. Samples (up to 80 λ) were subsequently applied 5 cm from the cathode using 5 x 10 mm strips of Whatman 3 MM chromatography paper. IEF was performed overnight at a constant voltage of 850 V across the length of the gel at 12°C. Cathodal and anodal electrode solutions were 1 M NaOH and 1 M H_3PO_4, respectively. An LKB Multiphor apparatus was used for all IEF experiments. Isozyme patterns 1-2 and 2-2 were confirmed using affinity starch gel electrophoresis, as described (18). The isozymes were visualized using a 4MU α-Glc filter paper overlay in the native electrophoretic systems described.

3. Western blotting: Western blotting following SDS-PAGE and IEF was performed as suggested by the manufacturer (Bio-Rad Labs, Richmond, CA). Transfer buffers were 25 mM Tris, 192 mM glycine, 10% (v/v) methanol, pH 8.3, and 0.7% acetic acid for SDS and IEF gels, respectively. All transfers were performed at 4°C. The transferred proteins were immunostained with rabbit PC Abs or MC Abs to placental GAA, followed by biotinylated anti-rabbit IgG and avidin DH and horseradish peroxidase H (Vector Laboratories, Inc., Birlingame, CA). The transferred coupled proteins were visualized by staining for peroxidase using HRP Color Development Reagent (Bio-Rad Laboratories, Richmond, CA).

4. Rocket Immunoelectrophoresis: Rocket antigen-antibody electrophoresis was performed essentially as described (41). Fibroblasts, resuspended in water or in 0.05 M citrate/phosphate buffers of varying pH, were lysed by sonication (5 cycles of 10 s duration) using a Branson sonifier, model 200, with a cup horn attachment (Branson Sonic Power, Danbury, CT). The lysates were centrifuged at 12,000 g for 20 min and the supernatants collected and assayed for enzyme activity and protein. Rocket immunoelectrophoresis was performed on 6.5 x 8.0 cm plastic plates. A 1% agarose (Seakem, Marine Colloids Div., FMC Corp., Rockland, ME) solution was prepared in 0.06 M Tris-sodium barbital buffer, pH 8.8 (Gelman high resolution buffer, Gelman Instruments, Ann Arbor, MI); 8 x 2 cm "bridges", each containing 4.0 ml of the agarose, were placed at the anode and cathode ends of the plate. The center of the plates (2.5 x 8 cm) was filled with 4.2 ml agarose containing 8 µl (approximately 80 µg protein) of partially purified rabbit anti-GAA Abs. Wells were cut at the cathode end of the Ab-containing gel and were filled with 10-25 µl of sample. Electrophoresis (bridge buffer same as the gel buffer) was carried out at 25 mAmp for 4 h at room temperature in a horizontal electrophoresis cell (model 1415, Bio-Rad, Richmond, CA). The gel was subsequently washed in saline for 8-12 h. The Ab-containing portion was overlaid with 0.3 ml of a 1:3 dilution of sheep anti-rabbit peroxidase conjugated IgG (Cappel, Cochranville, PA). The plate was incubated at room temperature for 8-12 h in a humidified chamber. After another 8-12 h wash, the gel was stained with a solution containing 25 mg diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) and 0.15 ml 3% H_2O_2 in 0.1 M Tris buffer, pH 7.6

(56). After 30 min of staining, the rockets were easily visualized using an indirect light source. The plates were magnified on a viewbox and the height of the rocket, from the top rim of the well to the the top of the precipitin arc, was recorded.

F. Enzyme Digestion:

1. Trypsin digestion: Trypsin digestion of homogeneous GAA was performed as follows. One-ninth volume of ice cold trichloroacetic acid was added to 1.5 ml microfuge tubes containing 4-10 nmol of electro-eluted enzyme. After 30 min at 4°C, the samples were centrifuged for 10 min at 10,000 x g, and the precipitate washed twice with cold acetone. After drying (either at room temperature or under a gentle stream of nitrogen), the protein was dissolved in 50 µl of fresh 8 M urea and adjusted to pH 8.0 by the addition of NH_4HCO_3. TPCK-Trypsin (1 mg/ml in 1.0 mM HCl) was mixed with the GAA (1 µg trypsin/25 µg GAA), the reaction mixture was incubated at 37°C for 24 h and then terminated by immersing the tube in an acetone/dry ice bath. The resultant peptides were purified by reversed phase HPLC as described below.

2. V8-digestion: Isozymes 1, 2 and 4 of GAA, purified by chromatofocusing, were digested with S. aureus V8 protease (Miles Scientific, Naperville, IL). This protease cleaves proteins on the carboxyl side of Asp and Glu. Briefly, 1 µg of purified enzyme protein was boiled for 2 min in Tris/HCl, pH 6.8, containing SDS, glycerol and Bromophenol Blue. The mixture was cooled to room temperature, V8 protease was added and the protein digested for 2.5 h at 37°C. Following this incubation, SDS in β-ME (final concentration 1.6% SDS in 10% β-ME) was added, the mixture was boiled for an additional 2 min, after which time the

reaction mix was frozen at -70°C until analyzed. Final concentration of buffer was 0.125 M Tris/HCl, 0.5% SDS, 10% glycerol and 0.001% Bromophenol Blue. Under these experimental conditions, consistent partial or complete digests were obtained at ratios 1:1 and 15:1 (V8:GAA), respectively. The digests were electrophoresed on 12.5% SDS polyacrylamide gels (53), Western blotted and immunostained with the MC Abs and PC Abs. The peptides reacting with the PC and MC Abs were located using an indirect avidin-biotin immunoperoxidase staining procedure.

3. N-Glycanase^{TM} digestion: N-Glycanase^{TM} (peptide:N-glycanase-F) digestion of GAA was performed as suggested by the manufacturer (Genzyme Corp., Boston, MA). The enzyme protein (0.5-1.0 µg) was boiled for 3 min in the presence of 0.5% SDS and 0.1 M β-mercaptoethanol. The sample was then diluted with sodium phosphate buffer, pH 8.6, EDTA and NP-40 (final concentrations: 0.2 M sodium phosphate, 5 mM EDTA, 1.25% NP-40). N-glycanase^{TM} (0.5 to 1.0 units) was then added (final concentration: 10-20 units/ml) and the reaction mixture was incubated for 24-48 h at 30°C. The reaction was stopped by rapid freezing. Digested samples were stored at -80°C until analyzed.

4. Neuraminidase treatment: Crude placental or purified enzyme (20-100 µl; 10-200 µg) were mixed with 25-100 µl neuraminidase from *Vibrio cholerae* (Calbiochem-Behring, LaJolla, CA); 1 unit/ml) in 0.7 mM citrate/phosphate buffer, pH 4.8. The reaction mix was incubated for 17-19 h at room temperature. The reaction was terminated by rapid freezing and stored at -80°C until analyzed.

5. Phosphatase treatment: Aliquots of acid and alkaline phosphatase (Sigma Chemical Co., St. Louis, MO), supplied as ammonium sulfate suspensions, were centrifuged for 15 min at 10,000 x g. The pelleted precipitate was resuspended in 0.1 M Tris/HCl, pH 7.4 (alkaline phosphatase) or in 0.05 M acetate buffer, pH 5.0 (acid phosphatase). GAA protein was added and the reaction was incubated at 37°C for 2 h. The reaction was stopped by rapid freezing. The digested samples were subsequently analyzed by IEF.

G. Amino Acid Composition and Microsequencing:

Amino acid sequence and composition analyses of the electroeluted GAA samples were performed by Dr. Kenneth Williams of the Protein Chemistry Facility in the Department of Biophysics and Biochemistry at Yale University

H. HPLC of Tryptic Peptides:

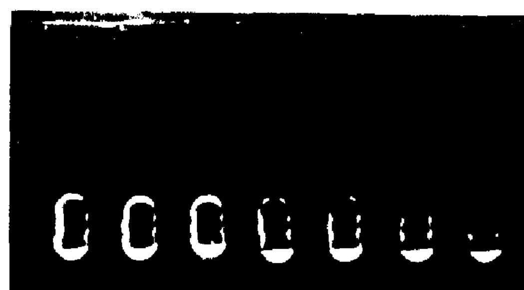
GAA tryptic peptides were resolved by chromatography on a 5 μ Vydac C_4 reversed phase column (4.6 mm i.d. x 25 cm) which had been equilibrated with 100% water with 0.05% trifluoroacetic acid (solution A). Sequential linear gradients of 0-60% acetonitrile (solution B; 80% acetonitrile, 20% water with 0.05% trifluoroacetic acid) from 0-180 min and 60-80% acetonitrile from 180-240 min were used to separate the peptides. The column was developed at a flow rate of 0.7 ml/min. The eluate was monitored by absorbance at 214 and 280 nm. For each HPLC analysis, 12-22 μg digested enzyme was injected.

V. RESULTS AND DISCUSSION

A. Rocket Immunoelectrophoresis of the Residual Enzyme Proteins and Isozyme 2 in Cultured Skin Fibroblasts.

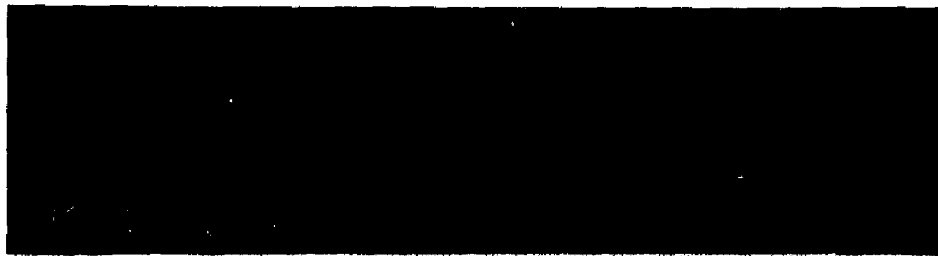
Structural differences between the normal and mutant enzyme proteins in crude fibroblast lysates were demonstrated in a rocket immunoelectrophoretic system, using monospecific rabbit Abs raised against purified placental GAA. Fibroblast lysates were prepared by sonication in water, or in phosphate buffers, pH 4 through 8. The total amount of enzyme activity recovered under these varying conditions was virtually identical, although consistently higher specific activities were obtained in lysates prepared at pH 4 and 5, i.e., more specific enzyme protein was recovered per mg protein in lysates prepared in acid buffer. This increase in specific enzyme protein in lysates prepared in acid buffer was clearly demonstrated by rocket immunoelectrophoresis. When a constant amount of lysate protein was applied to the gel, consistently higher rockets were formed with lysate prepared in acid buffer than with lysates prepared in water (see Fig. 2). When enzyme preparations from normal fibroblasts were subjected to rocket immunoelectrophoresis, immunoprecipitin arcs formed regardless of the preparative conditions. In contrast, the enzyme in preparations from CRM-positive infantile (M-4581), juvenile (J.N.) and adult (M-3773; GM-443) subtype fibroblasts failed to form a rocket when prepared in water, or in buffers at pH 6 through 8 (Fig. 1). When prepared at pH 4 and 5, rockets formed whose heights were directly proportional to the amount of enzyme activity applied. Of interest was the finding that the enzyme protein in the CRM-positive infantile subtype (M-4581) fibroblasts,

Figure 1: Rocket immunoelectrophoresis of adult subtype fibroblast lysates prepared in phosphate buffer and in water. Well 1, control lysate prepared in water (10 µg); wells 2 to 6, adult subtype (M-3773) lysates prepared in phosphate buffer, pH 4, 5, 6, 7 and 8, respectively (25 µg); well 7, adult subtype lysate prepared in water. Rockets were obtained with lysates prepared at pH 4 and 5, but not with lysates prepared at pH 6 through 8, or in water. The normal lysate prepared in water showed no loss in immunoreactivity.



1 2 3 4 5 6 7

Figure 2: Rocket immunoelectrophoresis of lysates from normal and mutant fibroblasts prepared in water and in phosphate buffer, pH 5. Wells 1-6, lysates prepared in water; wells 7-12, lysates prepared in buffer; wells 1 and 7, CRM-negative infantile subtype (GM-244; 25 µg); wells 2 and 8, CRM-positive infantile subtype (M-4581; 25 µg); wells 3 and 9, adult subtype (M-3773; 25 µg); wells 4 and 10, juvenile subtype (J.N.; 25 µg); wells 5 and 11, a cell line homozygous for isozyme 2 (20 µg); and wells 6 and 12, control (10 µg).


1 2 3 4 5 6 7 8 9 10 11 12

lysed at pH 4 or 5, consistently formed an atypical, less dense rocket than the control or the other mutant enzymes (Fig. 2).

Further experiments indicated that the mutant enzymes differed, not only compared to the normal enzyme, but also compared to each other. When the substrate maltose was added to the Ab-containing gel, the anodal migration of all of the enzyme forms was increased to the same extent. The rockets that formed were lifted away from the application wells. The quality of the immunoprecipitin arc formed by the normal enzyme was not altered. In contrast, the density of the immunoprecipitin arc formed by the CRM-positive infantile (M-4581) as well as the juvenile (J.N.) form enzyme was reduced, resulting in a very weak rocket. The enzyme from the adult subtype (M-3773) lost the ability to form a rocket almost entirely.

The immunologic properties of isozyme 2 paralleled those of the normal GAA isozyme 1. The mutant enzyme protein was immunoreactive whether prepared in water or in phosphate buffers, pH 4 through 8 (Fig. 2). Its anodal migration in the presence of maltose increased to the same extent as that of the normal, juvenile and CRM-positive infantile form enzyme. However, the quality of the immunoprecipitin arc was not affected. The reduced affinity of the Type 2 isozyme for starch was also demonstrated in the rocket immunoelectrophoretic system. When the isozyme was electrophoresed through a strip of starch-containing agarose, it was only slightly retarded and formed rockets of normal height and shape as it migrated into the Ab-containing portion of the gel. In contrast, the normal enzyme was severely retarded in the starch strip and either formed partial, unclosed rockets or no rockets under

the experimental conditions used. The immunologic properties of the different subtypes and isozymes are summarized in Table 1.

These studies demonstrated that each of the residual enzyme proteins, whether catalytically active or inactive, cross-reacted with PC, monospecific anti-GAA Abs against the purified placental enzyme. Furthermore, results indicated that GAA isozymes 1 and 2 shared immunologic identity. However, structural differences affecting immunoreactivity were demonstrated among the residual enzyme proteins from the subtypes and variants of glycogenosis II.

The reduced immunoreactivity of the catalytically inactive enzyme protein in the CRM-positive infantile subtype (M-4581) fibroblasts indicated that the number and/or nature of the antigenic determinants differed from that of the normal enzyme. The loss of immunoreactivity of this variant enzyme, as well as the residual enzyme proteins in the adult (M-3773) and juvenile (J.N.) subtype fibroblasts, when prepared in water or in buffers of pH 6 through 8, suggested an increased pH lability of these enzyme forms. Indeed, studies have shown that GAAs, purified from normal and adult subtype fibroblasts, were heat-inactivated more rapidly at pH 7.0 than at pH 4.0 (38). No difference in the rate of inactivation was observed between the normal and mutant enzymes. However, the purified enzymes were stabilized by the addition of high concentrations (0.8 mg/ml) of BSA. It is likely that the combination of an unfavorable pH during the preparation of the lysates followed by prolonged electrophoresis (4-5 h) at pH 8.8, destroys the immunologic integrity of the mutant residual enzymes. Although a pH of 8.8 destroys the enzymatic activity of the normal enzyme, it may not be sufficiently high to denature the enzyme to a point where it is immunologically

unreactive. Alternatively, the pH-dependent loss of immunoreactivity may be a function of the lower enzyme concentration in fibroblast lysates from the CRM-positive mutations. However, when normal cell lysates were diluted, either with buffer or with a CRM-negative lysate, rocket formation still took place. Therefore, results of these studies indicated that the altered immunoreactivity of the residual enzyme protein in the CRM-positive infantile (M-4581), the juvenile (J.N.) and the adult subtype (M-3773; GM-443) fibroblasts were due to structural differences.

The studies also showed that the immunologic properties of the residual enzyme proteins were altered when immunoelectrophoresed in the presence of the substrate, maltose. These findings suggested that binding of substrate molecules induced conformational changes which resulted in decreased immunoreactivity of the mutant enzyme proteins. The data further suggested that the CRM-positive infantile subtype (M-4581) enzyme had the same binding properties for maltose as the normal enzyme, since the change in anodal migration of the two enzyme forms was the same in the presence of this substrate.

B. Studies of the Metabolism of GAA in Fibroblasts.

The reduced levels of enzyme activity and protein in the various subtypes of glycogenosis could result from differences in the metabolism of GAA. Therefore, experiments were designed to assess the turnover of the mutant enzymes.

1. Enzyme Activity and Protein in Relation to the Stage of Cell Growth: Determination of enzyme activity and quantitation of the amount

of enzyme protein by rocket immunoelectrophoresis in normal fibroblasts at different stages of cell growth showed that the enzyme level increased 1.5- to 2-fold by late confluency over early confluency values. A similar increase in activity and enzyme protein was observed in adult (M-3773) and juvenile (J.N.) subtype fibroblasts. No increase in the apparent residual GAA activity was detected in the CRM-positive infantile subtype (M-4581) fibroblasts (Fig. 3). The amount of enzyme protein, however, did increase with time in culture, showing the same 1.5- to 2-fold elevation over early confluency values as with control and with the other CRM-positive mutant fibroblasts (Fig. 4). This finding suggested that the small amount of enzyme activity measured in the infantile subtype fibroblasts was not actually GAA activity, but was probably the result of non-specific hydrolysis of the substrate. At no time was enzyme protein detected in the CRM-negative adult (GM-1935) or the CRM-negative infantile subtype fibroblasts under the culture conditions used (see Table 1).

Studies concerning the amount of GAA at different stages of cell growth suggested that the enzyme synthesis in cells homozygous for the isozyme 2 (phenotype 2 cells) differed from that in normal, adult, juvenile and CRM-positive infantile subtype fibroblasts. In the phenotype 2 fibroblasts, enzyme activity and protein increased rapidly, and after the cells reached late confluency, obtained levels that were 4- to 6-fold greater than at early confluency (Table 2). The GAA activity in phenotype 2 fibroblasts at early confluency was reduced to 24% of that in normal type 1 fibroblasts when maltose was the substrate, and to 5% of normal when glycogen was the substrate. In two fibroblast lines derived from unrelated patients with the adult subtype of GAA deficiency

Figure 3: GAA activity in normal and mutant cultured skin fibroblasts from early to late confluency. Adult subtype: M-3733; juvenile subtype: J.N.; CRM-positive infantile subtype: M-4581.

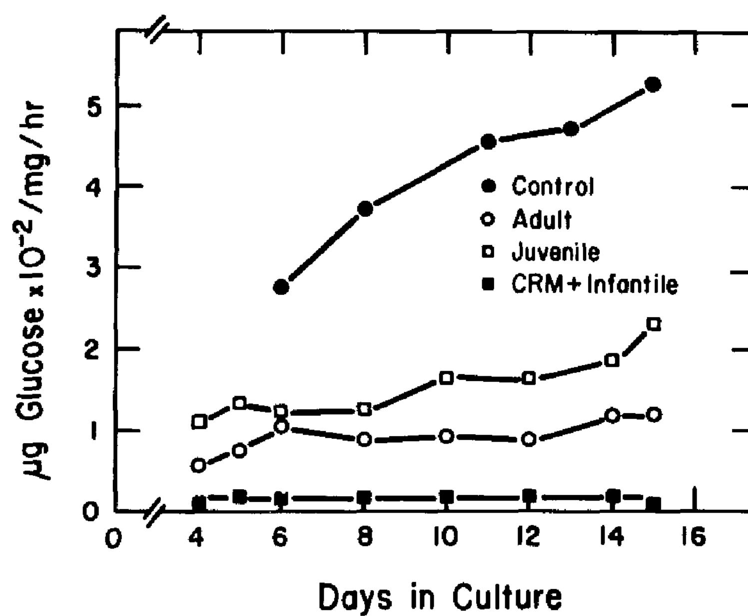

ACID α-GLUCOSIDASE ACTIVITY WITH TIME
IN CULTURE
μg Glucose x10⁻²/mg/hr
Days in Culture
Control
Adult
Juvenile
CRM+ Infantile
0
1
2
3
4
5
0
4
6
8
10
12
14
16

TABLE 1

Immunologic Properties of GAA Isozymes 1 and 2 and the Residual Enzyme Proteins in Glycogenosis II Fibroblasts

Fibroblast Source	Enzyme Activity	Enzyme Protein	Ab Binding pH 5	Ab Binding pH 7	Substrate Specificity
	(% of Control)				
Isoyzme 1[a]	100%	100%	+	+	M+[d]; G+[e]
Isozyme 2[b]	20 - 60%[c]	20 - 60%[c]	+	+	M+ ; G-
Infantile Subtype					
CRM-neg.	1 - 3%	-	-	-	-
GM-244					
GM-248					
GM-4912					
GM-3329					
WG-482					
WG-173					
WG-285					
M-2478					
B-4					
C-482					
CRM-pos.	1 - 3%	30 - 50%	weak	-	M+ ; G+
M-4581					
Juvenile Subtype	16 - 28%	12 - 28%	+	-	M+ ; G+
J.N.					
Adult Subtype					
CRM-neg.	1 - 3%	-	-	-	-
GM-1935					
CRM-pos.	9 - 22%	16 - 22%	+	-	M+ ; G+
GM-443					
M-3773					

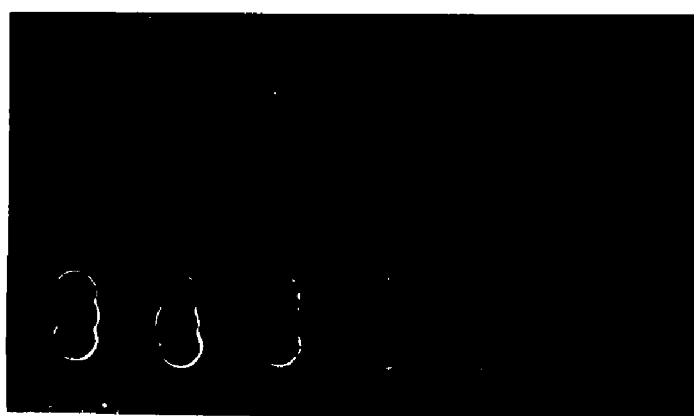
[a]Normal fibroblasts were from 16 unrelated subjects tested for heterozygosity of various metabolic disorders and found to be normal.

[b]A fibroblast strain homozygous for GAA isozyme 2.

[c]Residual enzyme activity and protein observed at early and late confluency, respectively.

[d]M = maltose; [e]G = glycogen

Figure 4: GAA protein in cultured skin fibroblasts harvested at early and late confluency. Wells 1 and 2, CRM-negative infantile subtype (GM-244) harvested at early and late confluency, respectively; wells 3 and 4, CRM-positive infantile subtype (M-4581) harvested at early and late confluency, respectively; wells 5 and 6, normal control harvested at early and late confluency, respectively. Wells 1-4 contain 25 μg protein; wells 5 and 6 contain 10 μg protein.



1 2 3 4 5 6

TABLE 2

GAA Activity in Cultured Fibroblasts Harvested at Early and Late Confluency

Fibroblast Strain	Acid α-Glucosidase Activity[a]					
	Early Confluency		Late Confluency		Ratio: Late/Early	
	Maltose	Glycogen	Maltose	Glycogen	Maltose	Glycogen
Type 1	247.5±107.7[b]	129.2±56.2[b]	518.3±172.5[b]	253.0±72.5[b]	2.09	1.96
Type 2	58.2[c]	6.4[c]	310.8[c]	39.0[c]	5.3	6.09
Adult Subtype M-3773	19.0	9.6	39.3	19.4	2.05	2.02
Type 1-2 M-3405	85.6	30.1	187.0	64.7	2.18	2.14

[a]μg glucose/mg protein/h

[b]mean±SD; n=7

[c]mean of three different preparations

(M-3773; GM-443), the activity was approximately 8% of normal with both substrates. At late confluency, the enzyme activity increased in all cases studied. However, type 2 fibroblasts showed the greatest relative increase in GAA activity, increasing to 60% of normal when maltose was the substrate and to 15% of normal when glycogen was the substrate. In the two adult GAA deficient fibroblast lines, the enzyme activity increased in parallel with that in normal fibroblasts and, therefore, remained at 8% of normal activity. The activities of three other lysosomal enzymes in phenotype 2 fibroblasts, α-L-fucosidase, β-D-glucuronidase and β-D-galactosidase, were within the normal range.

2. Turnover of GAA: To determine the turnover rates of the normal and mutant enzyme proteins, fibroblast cultures were treated with 30 μg/ml cycloheximide, and enzyme activity and protein were followed for up to 5 days. This concentration of cycloheximide inhibited protein synthesis by more than 95%. No decrease in enzyme activity was observed in any of the cases studied. On the contrary, the specific activity increased slightly over the 5 days following cycloheximide treatment (Fig. 5). This increase is most probably due to a general reduction in cellular protein relative to GAA. These findings indicate that GAA is very stable in the intracellular environment in both normal and mutant fibroblast strains. When the enzyme protein from cycloheximide-treated normal and mutant cells was examined by rocket immunoelectrophoresis, no change in the quality of the rockets was observed (Fig. 6 A-D). The proportionality between enzyme activity and the amount of enzyme protein detected was retained over the entire 5 day period, despite the rapid deterioration of the treated cells. The catalytically inactive enzyme

Figure 5: GAA activity in normal and mutant cultured skin fibroblasts after cycloheximide treatment. A slight increase in specific activity was observed in all fibroblasts examined. Adult subtype: M-3773; juvenile subtype: J.N.; CRM-positive infantile subtype: M-4581.

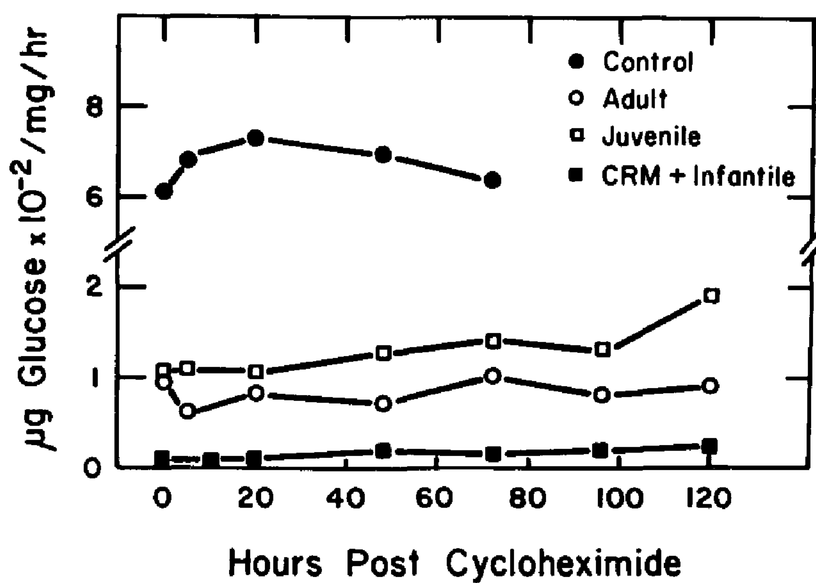
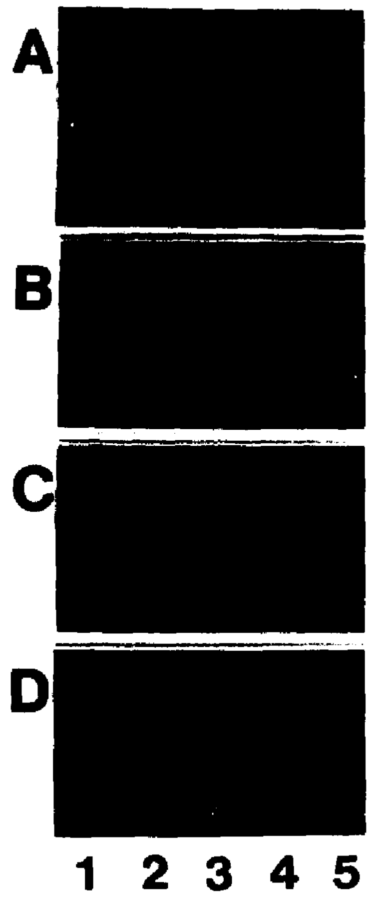

ACID α-GLUCOSIDASE ACTIVITY AFTER
ADMINISTRATION OF CYCLOHEXIMIDE
μg Glucose x 10^-2/mg/hr
Control
Adult
Juvenile
CRM + Infantile
0
1
2
6
8
0
20
40
60
80
100
120
Hours Post Cycloheximide

Figure 6: GAA protein in normal and mutant cultured skin fibroblasts after cycloheximide treatment. A, control lysates (10 µg); B, adult subtype (M-3773; 25 µg); C, juvenile subtype (J.N.; 25 µg); and D, CRM-positive infantile subtype (M-4581; 25 µg). Wells 1-5 correspond to lysates prepared from cells treated with cycloheximide for 1, 2, 3, 4 and 5 days. No deterioration in the quality of the rockets was observed.


A
B
C
D
1 2 3 4 5

protein in the CRM-positive infantile subtype (M-4581) fibroblasts was as stable as the enzyme protein in the normal, juvenile (J.N.) and adult subtype (M-3773) fibroblasts (Fig. 6D).

These findings suggested that the reduction in the amount of enzyme protein in the CRM-positive infantile, juvenile and the adult subtype fibroblasts did not result from an increased rate of degradation. However, these data do not rule out the possibility of a defect in the synthesis or processing of the GAA protein in the various forms of the enzyme deficiency. In addition, since cycloheximide may also affect enzymes which normally would be involved in the degradation of GAA, experiments using radiolabeled tracers to study the turnover of the enzyme proteins are indicated.

C. MC Abs to GAA:

1. Production of MC Abs to GAA: The antigenic properties of the various mutant enzyme forms described above have been characterized using heterogeneous rabbit anti-placental GAA Abs. This approach has permitted the identification and discrimination of the above mutant forms on the basis of their immunoreactivity (39,40). However, increased discrimination and characterization can be achieved by the use of MC Abs each of which recognizes only a single antigenic determinant (epitope). Therefore, a major effort was directed to produce MC Abs to GAA for the investigation of the biochemical and molecular genetic basis of the heterogeneity in glycogenosis II.

MC Abs to GAA were produced using the hyperimmunization procedure described by Stahli et al. (49). Production of the hybridomas and the subsequent screening for anti-placental GAA Abs was carried out as described (50,51). To date, 19 Abs to GAA have been identified; twelve of these have been subcloned and are stable and have been successfully propagated in the ascitic fluid of mice.

These hybridomas were tested for the presence of GAA Abs using two different methods: a peroxidase ELISA which detects both neutralizing and non-neutralizing Abs, and a GAA ELISA, using the synthetic substrate 4MU α-Glc, which detects only the non-neutralizing Abs.

All twelve hybridomas were positive for the production of anti-GAA Abs using the peroxidase ELISA. Only ten of the clones were positive using the 4MU α-Glc ELISA. The two clones that were peroxidase-positive but 4MU α-Glc-negative are of particular interest. Presumably these MC Abs are directed against determinants at or near the substrate binding or catalytic site of the enzyme. These MC Abs should be extremely valuable for the examination of the residual enzyme protein in the CRM-positive infantile fibroblasts and in the different isozyme types.

2. Characterization of MC Abs to GAA: To date, twelve MC Abs to GAA have been characterized. All were of class IgG and represented the subclasses γ_1/λ, γ_1/k, γ_{2a}/k, and γ_{2b}/k. All twelve MC Abs recognized the native and the denatured forms of isozymes 1, 2 and 4. Two of the MC Abs, Sp2/52 and Sp2/53, were highly specific for GAA. Furthermore, these two MC Abs were shown to be directed against the active center (catalytic and/or binding site) of the human GAA molecule (see below). The properties of the MC Abs are summarized in Table 3. Because of their exquisite specificity, MC Abs Sp2/52 and Sp2/53 were used to char-

TABLE 3

Properties of MC Abs to Placental GAA

MC Ab	Isotype	Immuno-reactivity[a]	Cross-Reactivity[b]	Neutralization	
				Tube Pptn	ELISA
Sp2/51	$\gamma_1\lambda$	+	+	+	-
Sp2/52	$\gamma_1 K$	+	-	nd[c]	+
Sp2/53	$\gamma_{2a} K$	+	-	+	+
Sp2/54	$\gamma_1\lambda$	+	+	nd	-
Sp2/55	$\gamma_1\lambda$	+	+	nd	-
Sp2/56	$\gamma_1 K$	+	+	+	-
Sp2/57	$\gamma_{2b} K$	+	+	+	-
Sp2/58	$\gamma_1 K$	+	+	nd	-
Sp2/59	$\gamma_1 K$	+	+	nd	-
Sp2/60	$\gamma_1 K$	+	+	nd	-
Sp2/61	$\gamma_1 K$	+	+	nd	-
Sp2/62	$\gamma_1 K$	+	+	nd	-

[a]All MC Abs reacted with the native and denatured enzyme forms of isozymes 1, 2 and 4.

[b]MV Abs indicated "+" reacted with cellular proteins other than GAA. Cross-reactivity was more pronounced with denatured than with native proteins.

[c]Not determined

acterize the isozyme forms in crude placental homogenates, and normal and deficient fibroblast lysates (see Section V.D.).

a. Epitope Specificity of MC Abs toward Proteolytic Digests of GAA Isozymes: Isozymes 1, 2 and 4 of GAA, purified by chromatofocusing (see Section V.E.2), were digested with *S. aureus* V8 protease (Miles Scientific, Naperville, IL). This protease cleaves proteins on the carboxyl side of Asp and Glu. The digests were electrophoresed on 12.5% SDS polyacrylamide gels (53), Western blotted and immunostained with the MC Abs and PC Abs. The peptides reacting with the PC and MC Abs were located using an indirect avidin-biotin immunoperoxidase staining procedure.

Fig. 7 shows that MC Abs Sp2/52 and Sp2/53 have unique epitope specificities. In the completely digested preparations (lanes 1, Fig. 7 A and B), Sp2/52 recognized a peptide with a $M_r \sim 16$ kDa, whereas Sp2/53 reacted with a peptide of $M_r \sim 11.5$ kDa; the PC Ab reacted with a number of peptides with M_rs ranging from 11.5-16 kDa (Fig. 7 C). Fig. 7 also shows a difference in reactivity with a number of peptides in the partially digested preparations (lanes 2). Although the intensity ratios of immunoreactive peptides varied between the two isozymes, no peptide(s) unique to isozyme 1 (Fig. 7) or 2 (Fig. 8) was detected. The pattern of immunoreactivity with isozyme 4 peptides was indistinguishable from that of isozyme 1 (data not shown).

b. Epitope Specificity of MC Abs as Determined by Competition ELISA: To determine the spatial relationship of the epitopes recognized by MC Abs Sp2/52 and Sp2/53, competition ELISAs were performed. Results of these studies showed that the epitopes recognized by

Figure 7: Immunoblot of _S. aureus_ V8 protease-digested GAA isozyme 1. Protease-treated and untreated proteins were electrophoresed in 12.5% SDS-polyacrylamide gels, immunoblotted and stained using an indirect biotin-avidin procedure. Panel A, stained with MC Ab Sp2/52; Panel B, stained with MC Ab Sp2/53; Panel C, stained with PC Ab. Lane 1: total digests; lane 2: partial digests; lane 3: undigested enzyme. Each lane contains 1 μg GAA protein.

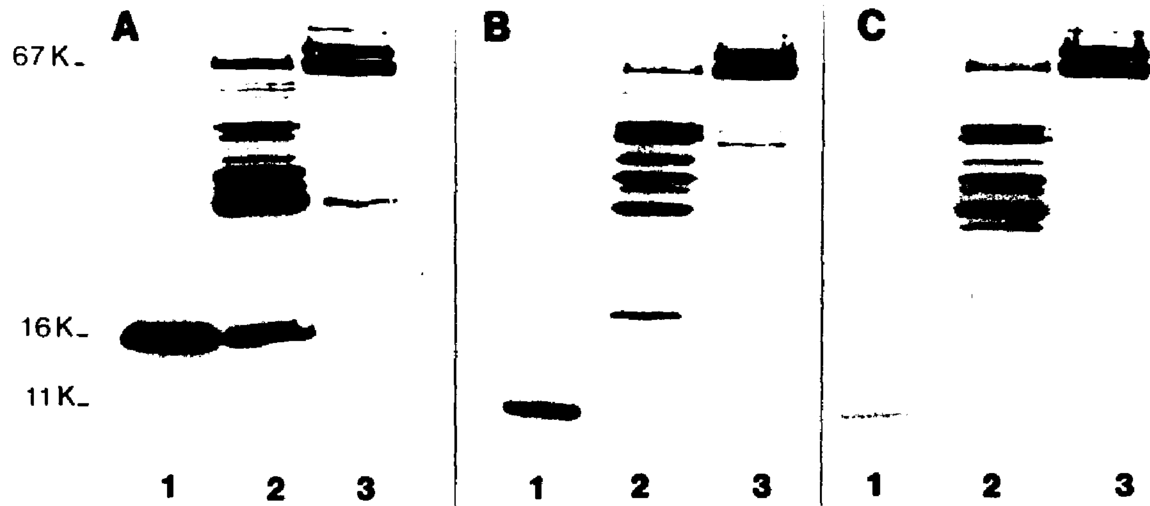
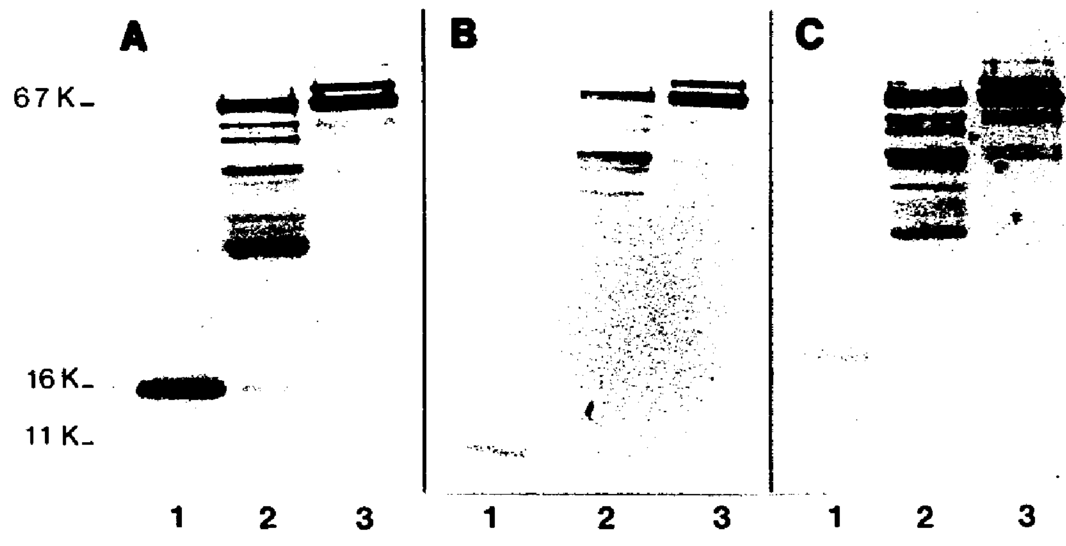

A
B
C
67K
16K
11K
1 2 3
1 2 3
1 2 3

Figure 8: Immunoblot of *S. aureus* V8 protease-digested GAA isozyme 2. For details, see Fig. 7.


A
B
C
67 K
16 K
11 K
1 2 3
1 2 3
1 2 3

Sp2/52 and Sp2/53 were in close proximity to each other. The Sp2/52 epitope appeared to have critical residues for binding near or overlapping that of the Sp2/53 epitope. The Sp2/53 epitope, on the other hand, appeared to have little or no overlap into the region of the Sp2/52 epitope. This interpretation was based on the finding that MC Ab Sp2/52 excluded Sp2/53 almost entirely, whereas MC Ab Sp2/53 was relatively inefficient in blocking further binding of Sp2/52. When both MC Abs were added simultaneously to GAA coated wells, the amount of Ab bound was much less than that observed by the sequential addition of the Abs. These data are summarized in Table 4. Collectively, the findings indicated that the Abs were competing for the same site(s) or sites in close proximity on the enzyme molecule.

Thus, these MC Abs are extremely valuable reagents in that they are directed toward the active site of the enzyme and in that they recognize distinct, but neighboring epitopes. Further studies with deglycosylated forms (see Section V.E.4) of the GAA isozymes demonstrated that the MC Abs were specific for the protein moiety of the enzyme and that the epitopes recognized did not include carbohydrate moieties.

c. Electrophoresis of Enzyme-MC Ab Complexes: The electrophoretic mobilities of three GAA-MC Ab complexes were evaluated in a native acrylamide gel system. The three MC Abs used were Sp2/56, Sp2/57 and Sp2/53. The two former hybridomas were positive for the production of Abs using the peroxidase and the 4MU α-Glc ELISA; the latter was peroxidase-positive but 4MU α-Glc-negative.

All three MC Abs completely inactivated GAA at dilutions of 0 and 1:2; Sp2/53 also inactivated the enzyme at a dilution of 1:4. At all

TABLE 4

Competition ELISA Between Active Site-Directed MC Abs

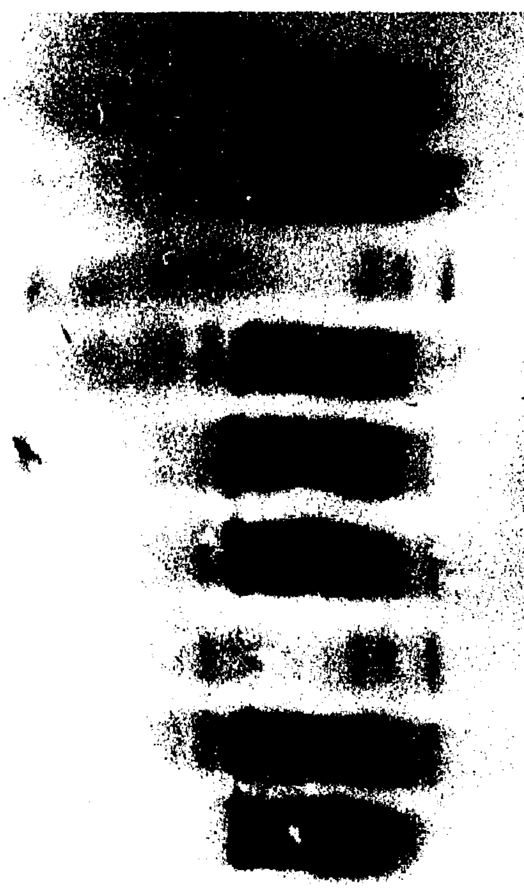
MC Ab	O.D. 420 nm	Percent Increase
Sp2/52	0.886	-
Sp2/53	0.729	-
Sp2/53, then Sp2/52	0.950	30.3[a]
Sp2/52, then Sp2/53	0.928	4.7[b]
Sp2/52 + Sp2/53	0.833[c]	-

[a]Increase relative to Sp2/53 binding.

[b]Increase relative to Sp2/52 binding.

[c]Binding observed was 51.4% of expected values (O.D. 1.62), assuming no competition.

Figure 9: IEF immunoblot of crude placental and fibroblast lysates immunostained with MC Ab Sp2/53. Note the three anodal proteins stained in the CRM-negative adult (GM-1935) and a CRM-negative infantile subtype (GM-248). A.E. is a patient with lysosomal storage of glycogen in the presence of normal GAA activity. Each lane contains 80 μg protein.


PLACENTA
NORMAL
CRM- ADULT
NORMAL
A.E.
NORMAL
CRM- INFANTILE
NORMAL
PLACENTA

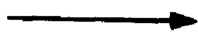
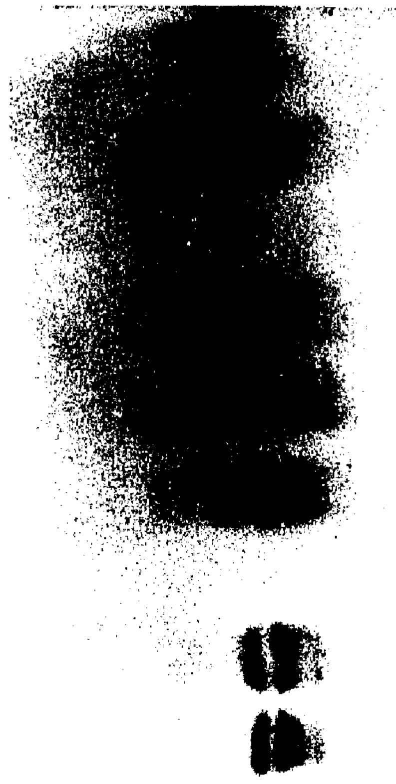

+

Figure 10: IEF immunoblot of crude placental and fibroblast lysates immunostained with PC Abs. Note the complete absence of staining in the CRM-negative infantile (GM-248) and the CRM-negative adult subtype (GM-1935). A.E. is a patient with lysosomal glycogen storage in the presence of normal GAA activity. J.E. is the father of A.E. Each lane contains 80 µg protein.



PLACENTA

NORMAL

CRM- INFANTILE

NORMAL

A.E.

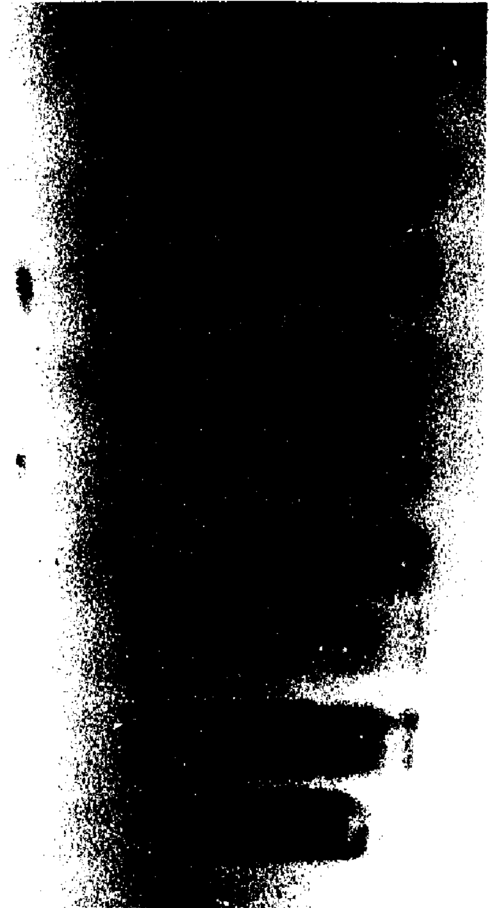
NORMAL

CRM- ADULT

NORMAL

J.E.

Figure 11: IEF immunoblot of crude placental and fibroblast lysates from CRM-positive mutations stained with MC Ab Sp2/53. Note CRM in the juvenile (J.N.) and adult (M-3773) subtypes, and the unique cathodal protein in the CRM-positive infantile subtype (M-4581). Each lane contains 80 µg protein.



PLACENTA
NORMAL
JUVENILE
NORMAL
ADULT
NORMAL
CRM+ INFANTILE
NORMAL
PLACENTA

+

other dilutions of antibody tested, only active, uncomplexed enzyme was observed on the gels. Because of the large amounts of enzyme necessary to perform these studies, the remaining nine MC Abs were not tested. These results indicated that these three MC Abs recognized the native enzyme forms of isozyme 1 and resulted in the formation of catalytically inactive enzyme-Ab complexes in solution.

D. GAA in Normal and Deficient Fibroblasts.

Although the MC Abs tested did not discriminate between the isozymes of GAA, they are, nevertheless, valuable reagents for elucidating the epitope composition of the normal and residual enzyme forms. Therefore, the MC Abs were used to examine the electrophoretic forms of GAA in fibroblasts from patients and variants with Pompe disease which previously were classified as CRM-positive and CRM-negative using PC Abs (38). Following IEF and Western blotting (57,58), approximately eight electrophoretic forms of GAA were observed in normal fibroblasts with both PC Abs and MC Abs Sp2/52 and Sp2/53. The enzyme forms focused between pH 4.39 and 4.70 and had enzymatic activity when stained with 4MU α-Glc. The MC Abs also recognized multiple enzyme forms in placental homogenates, which, in part, differed in electrophoretic mobility relative to the fibroblast forms.

MC Ab Sp2/53 was unique. It reacted with three anodal forms of GAA in lysates from the unique CRM-negative adult (GM-1935) as well as infantile subtype patient (GM-248) previously classified as CRM-negative using PC Abs (see Fig. 9). These proteins appeared to be authentic GAA due to their identical mobility with normal enzyme forms. However,

consistent with their previous classification as CRM-negative mutations, these enzyme forms were not detected using PC Abs (Fig. 10). The same three anodal forms also were identified by Sp2/53 in juvenile and adult subtype fibroblasts (see Fig. 11). Notably, the bands in the CRM-positive juvenile and adult patients had GAA activity. The total amount of GAA detected was greatly reduced relative to control fibroblasts, consistent with our previous findings of 10-22% residual enzyme activity and protein in juvenile and adult subtype fibroblasts, using PC Abs (38-41).

MC Ab Sp2/52, which recognized GAA in normal, adult and juvenile subtype fibroblasts, did not react with the anodal proteins in the CRM-negative adult (GM-1935) and CRM-negative infantile subtype (GM-248) fibroblasts.

The isozyme pattern in lysates from the CRM-positive infantile subtype (M-4581) was distinct from all other cases studied. The three anodal forms observed in the adult, juvenile and CRM-negative infantile subtypes were greatly reduced. In addition, a cathodal band, which was barely detected in normal and the other deficient lysates, strongly reacted with MC Ab Sp2/53 (Fig. 11); none of these enzyme forms had activity. This unique cathodal protein was also detected by MC Ab Sp2/52 and PC Abs.

The electrophoretic pattern of GAA in fibroblasts from an unusual patient (A.E.) with massive glycogen accumulation in the presence of elevated enzyme activity was virtually indistinguishable from that in controls (see Fig. 9). Thus, despite extensive biochemical and immunologic studies, it is still not known why this patient is affected so

severely. The defect may represent a mutation in the trafficking and/or compartmentalization of GAA.

To determine whether MC Ab Sp2/53, in addition to recognizing native enzyme forms in the CRM-negative mutations, also recognized the denatured enzyme protein, lysates were analyzed under denaturing conditions. Crude fibroblast lysates (30 μg) were denatured by heating for 5 min at 95°C in buffer containing SDS and β-mercaptoethanol. The denatured lysates were subjected to SDS-PAGE using a mini-gel apparatus. The proteins were electrophoretically transferred to nitrocellulose membranes and immunostained with MC Abs. MC Ab Sp2/53, indeed, recognized the denatured 67 kDa subunit of GAA in the CRM-negative mutations. The MC Ab also recognized a number of lower molecular weight forms in all of the lysates examined. In contrast, MC Ab Sp2/52 did not recognize either the 73 or the 67 kDa subunit of GAA in the CRM-negative mutations, but did react with the corresponding enzyme forms in a partially purified preparation of normal GAA, normal lysates and fibroblast lysates from the CRM-positive mutations. Furthermore, MC Ab Sp2/52 recognized more lower molecular weight forms than Sp2/53.

These experiments were also performed without β-mercaptoethanol, since there is evidence to suggest that disulfide bridges may play a role in antigenic determinant structure on the GAA molecule (25). No change was detected in the reactivity of the MC Abs to the high molecular weight forms of the enzyme in any of the cases tested. Notably, fewer lower molecular weight forms were observed in the absence of the reducing agent.

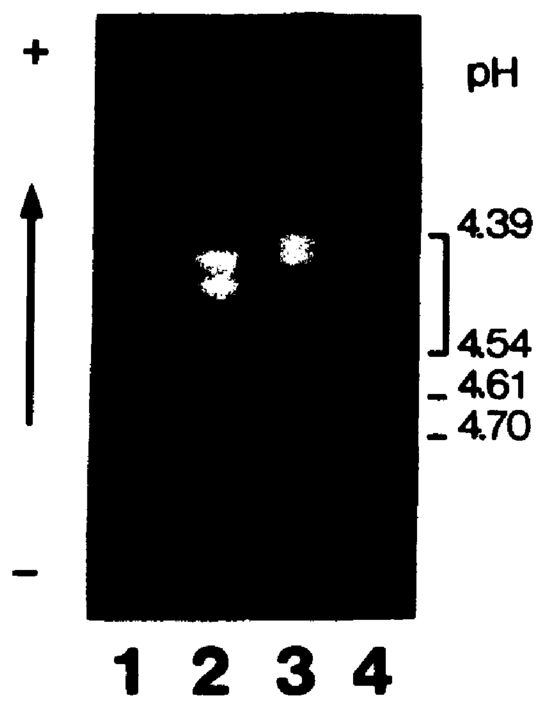
Thus, the use of MC Abs has demonstrated, for the first time, the presence of CRM to GAA in patients previously defined as CRM-negative using PC Abs.

E. Identification, Purification and Characterization of the Electrophoretic Forms of GAA Isozymes 1, 2 and 4:

To date, four isozymes, each encoded by a different allele at the GAA locus, have been identified. Furthermore, the existence of multiple electrophoretic forms of the enzyme, even in highly purified preparations, has been documented (59). Our prior demonstration that isozyme 2 did not hydrolyze starch as did the common isozyme 1, suggested that the other isozymes (i.e., 3 and 4) of GAA may have altered physical or kinetic properties which could be associated with disease. Therefore, detailed studies for the identification, purification and characterization of the multiple electrophoretic forms of GAA isozymes were performed to determine their relatedness, the basis of their charge heterogeneity and their involvement in the disease process. Toward that goal, an IEF system which resulted in the unequivocal identification of phenotypes 1-1, 1-2 and 1-4 in heterozygous placentae was developed. This system confirmed the occurrence of GAA electrophoretic forms which had pI values of 4.39 to 4.70 (isozyme 4, pI = 4.70; isozyme 2, pI = 4.61; isozyme 1, pI =4.54-4.39) (Fig. 12).

1. Screening of Placentae for Identification of the GAA Isozymes: Fresh placentae, obtained from the delivery room, and frozen placentae were used for these studies. The placental extracts were prepared and subjected to horizontal starch gel electrophoresis as described (26). Of

Figure 12: IEF of crude human placental homogenates. Lanes 1 and 4, phenotype 1-4; lane 2, phenotype 1-2; lane 3, phenotype 1-1. The gel was stained for enzyme activity with 4MU α-Glc. Each lane contains approximately 50 μg protein.


+
pH
4.39
4.54
4.61
4.70
−
1 2 3 4

214 placentae screened to date, 7 were found to have the isozyme pattern 1-2. No placenta with isozyme 3 was identified (19).

The same 214 placentae were rescreened by IEF. Seventeen had the isozyme pattern 1-4. Based on this sampling, the gene frequencies were calculated to be 0.942, 0.017 and 0.041 for alleles 1, 2 and 4, respectively. Phenotypes 2-2, 1-3, 3-3 and 4-4 were not observed. Gene frequencies calculated in a similar Canadian survey of 201 placental samples were 0.91, 0.03 and 0.06 for isozymes 1, 2 and 4, respectively (20). It appears that alleles 2 and 4 are rarer in the New York sampling and may reflect differences in the ethnic makeup of the population examined.

2. Purification of Isozymes 1 and 4 from a Heterozygous [1-4] Placenta: Isozyme 4 of GAA was partially purified from the heterozygous placenta using procedures developed in this laboratory (26). Since it was not known whether isozyme 4 would co-purify with GAA isozyme 1, all steps in the purification were monitored by IEF. The fact that isozyme 4 co-purified with isozyme 1, suggested that they have similar physicochemical properties.

Separation of isozymes 1 and 4 was accomplished using chromatofocusing (60,61). Enzyme eluted from the Sephadex G-100 column was applied to a column packed with polybuffer exchanger PBE 94. The column was developed using a pH gradient of 5 to 4 as described. All fractions were monitored for enzyme activity with 4MU α-Glc. A typical elution profile is shown in Fig. 13. The peak fractions from each isozyme were individually pooled as indicated, precipitated with ammonium sulfate to remove the polybuffer, resuspended in saline, dialyzed extensively and

Figure 13: Elution profile of GAA isozymes 1 and 4 on a chromatofocusing column. The column was developed over a pH range of 5 to 4. Fractions I through VI were individually pooled, as indicated. Fractions I and II, GAA isozyme 4; fractions III-VI, electrophoretic forms of GAA isozyme 1.

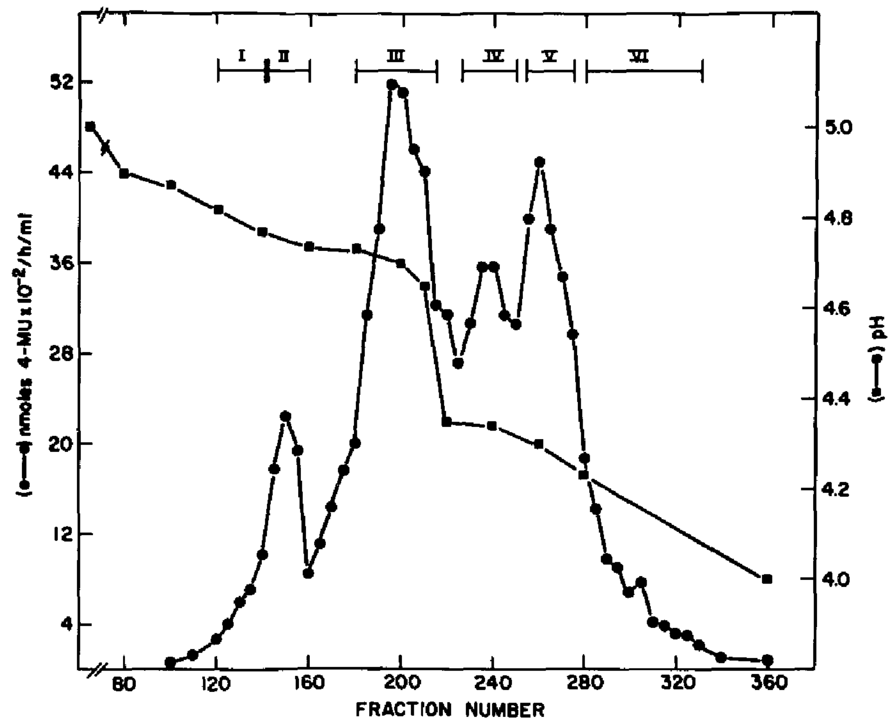
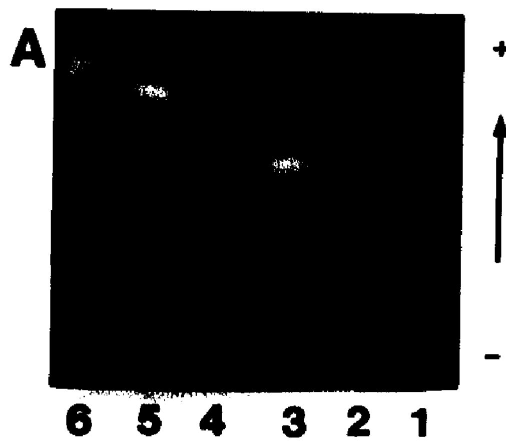

I
II
III
IV
V
VI
52
44
36
28
20
12
4
80
120
160
200
240
280
320
360
FRACTION NUMBER
5.0
4.8
4.6
4.4
4.2
4.0
(●—●) nmoles 4-MU x 10⁻²/h/ml
(■—■) pH

Figure 14: Electrophoretic forms of isozymes 1 and 4 isolated by chromatofocusing. Lanes 1 through 6 correspond to fractions I through VI in Fig. 13. A, the gel stained with 4MU α-Glc; B, immunoblot stained with MC Ab Sp2/53. .


A
+
-
6 5 4 3 2 1

B
+
-
6 5 4 3 2 1

stored at -80°C for further use. Analysis of the purified enzymes by IEF (Fig. 14A) showed complete separation of isozymes 1 and 4. Fraction I and II corresponded to GAA isozyme 4, whereas fractions III through VI represent the electrophoretic forms of GAA isozyme 1. Interestingly, the enzyme in fraction IV contained two electrophoretic forms, one of which was more basic than the protein in the preceeding fraction (see Fig. 14). This was a consistent finding in three separate purifications of the isozyme 1 complex.

3. Purification of the Electrophoretic Forms of GAA Isozyme 2 from a Heterozygous [1-2] Placentae: On the basis of the results obtained with isozymes 1 and 4, phenotype 1-2 in placentae was reexamined. Upon IEF, multiple enzymatically active forms were observed in crude placental homogenates over a pH range of 4.61-4.39. A characteristic band at pH 4.61 distinguished the 1-2 from the 1-1 and 1-4 phenotype (see Fig. 12). Isozyme 2 has been purified to near homogeneity (42). The purified enzyme migrates as a single band on starch gel electrophoresis and demonstrates the typical 67 and 73 kDa subunits on SDS-PAGE. On IEF, the DEAE-purified isozyme 2 was resolved into four electrophoretic forms, which overlapped, but were distinct from those of isozyme 1 (see Fig. 15). Subsequent to this finding, the partially purified enzyme was subjected to chromatofocusing as described for isozymes 1 and 4, and resulted in the complete separation and isolation of the four electrophoretic forms of isozyme 2 (see Fig. 16).

4. Comparative Studies of the Physical, Kinetic and Immunologic Properties of the Electrophoretic Forms of Isozymes 1, 2 and 4: The purified preparations of the electrophoretic forms of isozymes 1, 2 and

Figure 15: IEF immunoblot of GAA isozymes 1, 2 and 4. Lane 1, isozyme 4 purified by chromatofocusing; lanes 2 and 3, DEAE-purified isozyme 2; lane 4, Sephadex G-100 purified isozyme 1. Each lane contains 0.8-1.0 μg protein. The blot was immunostained with MC Ab Sp2/53.

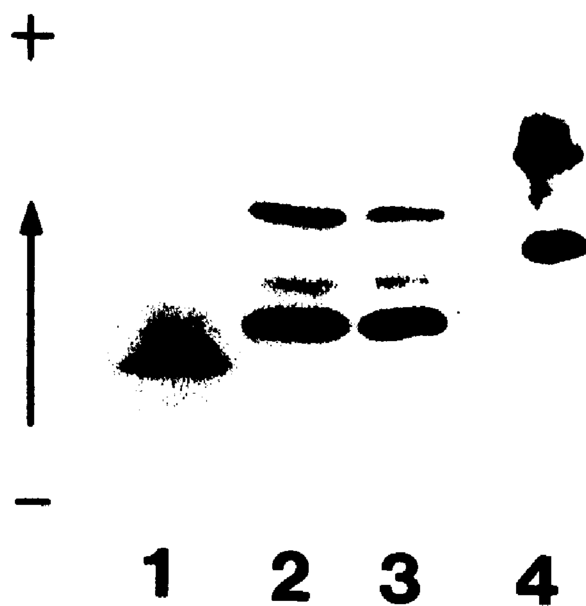
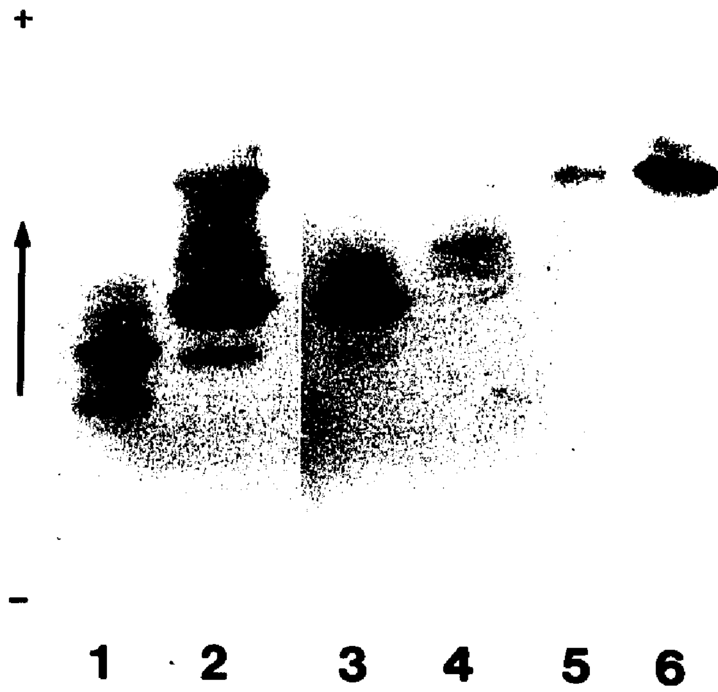

+
−
1
2
3
4

Figure 16: IEF immunoblot of the electrophoretic forms of GAA isozyme 2 purified by chromatofocusing. Lane 1, partially purified GAA isozyme 4; lane 2, DEAE-purified isozyme 2; lanes 3-6, the major electrophoretic forms of isozyme 2 eluted from a chromatofocusing column. Each lane contains 0.5-0.8 µg protein. The blot was immunostained with MC Ab Sp2/53.


+
-
1
2
3
4
5
6

4 obtained from heterozygous (1-2 and 1-4) placentae were analyzed for their physical, kinetic and immunologic properties. The preparations were examined for their subunit composition, pH optima, thermal stability, K_m and V_{max} (using maltose and glycogen as substrate) and for their immunoreactivity with PC and MC Abs.

a) Physical and kinetic properties: Comparative studies showed that the electrophoretic forms of isoyzmes 1 and 4 had the same pH optimum, thermostability, apparent K_m and V_{max} values toward maltose and glycogen, and similar K_is with the inhibitors, turanose, trehalose, α-methylglucoside and 1-deoxynojirimycin. In contrast, the electrophoretic forms of isozyme 2 all exhibited a reduced ability to catalyze glycogen and the atypical Michaelis-Menten kinetics characteristic of this isozyme, but otherwise shared the physicokinetic properties of isozymes 1 and 4 (see Table 5).

b) Subunit composition: The six enzyme fractions from a 1-4 placental preparation eluted from the chromatofocusing column were analyzed by SDS-PAGE to determine their subunit composition. As shown in Fig. 17, the enzyme forms in all six fractions contain a 67 kDa and a 73 kDa protein, although in varying proportions. The enzymes in fractions I and II (GAA isozyme 4) were composed of almost entirely the 73 kDa subunit. Fractions III through VI (the major electrophoretic forms of GAA isozyme 1) had progressively less of the 73 kDa subunit and a simultaneous increase in the 67 kDa subunit. All six fractions contained a low MW ($\cong$ 20 kDa) form which copurified with isozymes 1 and 4. That all of these denatured forms, including the 20 kDa form

TABLE 5

Physical and Kinetic Properties of GAA Isozymes 1, 2 and 4

Property	Isozyme 1[a]	Isozyme 2[b]	Isozyme 4[c]
pH Optimum	4.2	4.0 - 4.4	4.4
Thermal Stability		same	
Apparent K_m:			
Maltose (mM)	16 - 20	13 - 22	17
Glycogen (mg/ml)	41	-[d]	46
Apparent V_{max}:			
(µg Glu x 10^{-6}/h/mg)			
Maltose	11.9 - 12.4	1.5 - 2.7	6.6
Glycogen	7.4 - 7.8	-	2.8 - 4.4

[a]Chromatofocusing fractions III-VI; Fig. 14, lanes 3-6

[b]Chromatofocusing fractions I-IV; Fig. 16, lanes 3-6

[c]Chromatofocusing fractions I and II; Fig. 14, lanes 1 and 2

[d]All four electrophoretic forms demonstrated atypical Michaelis-Menten kinetics and a reduced ability to catalyze glycogen. Maltose:glycogen ratio were 11.2-12.1 compared to ratios of 1.0-1.9 with isozyme 1.

Figure 17: Subunit composition of the electrophoretic forms of GAA isozymes 1 and 4. S, standards; lanes 1-6 correspond to fractions I through VI in Fig. 13. Each lane contains 1 μg protein. The 10% acrylamide gel was stained with Coomassie Blue. Note the 20 kDa protein which co-purified with each electrophoretic form of isozymes 1 and 4.

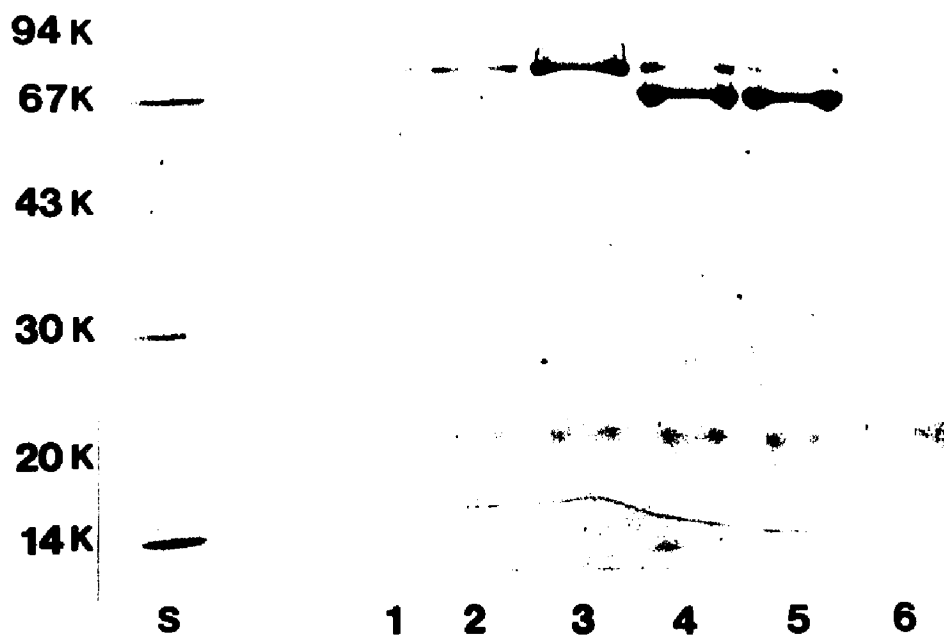
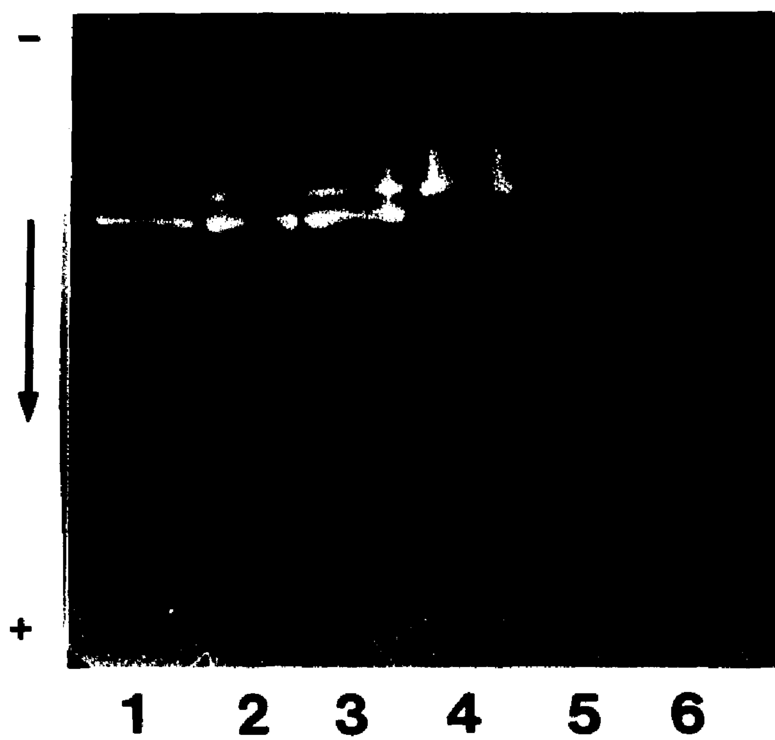

94 K
67K
43 K
30 K
20 K
14 K
S
1
2
3
4
5
6

Figure 18: SDS-PAGE of the electrophoretic forms of GAA isozymes 1 and 4 stained with fluoresceinated Concanavalin A. Lanes 1-6 correspond to fractions VI-I of Fig. 13. Note the low MW glycoprotein ($\sim$ 20 kDa) which co-eluted with each GAA enzyme form. Each lane contains 1 μg protein.


–
+
1
2
3
4
5
6

were glycoproteins was demonstrated by staining the gel with fluorescein-conjugated Concanavalin A (see Fig. 18).

Earlier studies showed that bovine and human liver GAA (110 kDa) were dissociated into 25-27 kDa forms in the presence of guanidine-HCl (62,63). However, recent studies by Martiniuk, Honig and Hirschhorn (64) demonstrated that the low molecular weight (∿ 20 kDa) protein bands in SDS-gels do not correspond to the guanidine-HCl generated low molecular weight species previously reported (62,63). When the I^{125}-labeled, guanidine-dissociated 20 kDa form was analyzed on SDS-gels, only a 64 kDa protein was identified. These findings suggested that mature GAA protein is made up of polypeptides which are bonded in the native state by at least two different types of interactions, one sensitive to SDS-denaturation and one sensitive to guanidine-HCl, but not SDS.

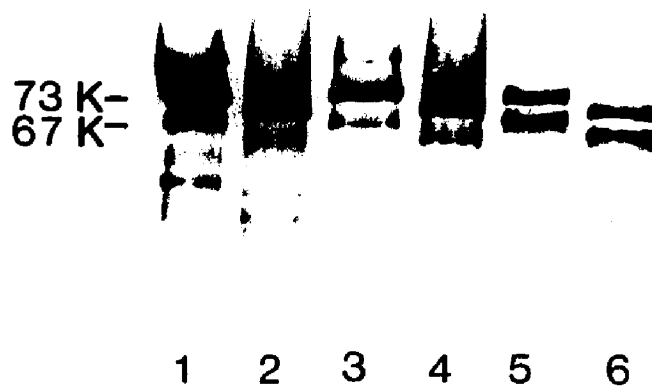
The finding that the 20 kDa glycoprotein co-purified with isozymes 1 and 4 throughout the purification procedure indicates that the protein is tightly associated with the enzyme. Indeed, one can speculate that this protein must be "buried" within the native enzyme structure. Furthermore, this tight association with GAA suggests that the 20 kDa protein may have physiologic significance, perhaps as an activator or stabilizing factor.

Analysis of the isozyme 2 chromatofocusing fractions by SDS-PAGE showed a similar trend from predominantly a 73 kDa to a 67 kDa subunit composition. The more basic enzyme protein corresponds to the 73 kDa subunit, whereas the more acidic enzyme forms were composed almost entirely of a 67 kDa subunit.

c) Immunologic properties: Comparative studies showed that the electrophoretic forms of isozymes 1, 2 and 4 were antigenically indistinguishable on the basis of their reactivity with PC and the 12 MC Abs under denaturing and non-denaturing conditions (see Fig. 14B). Further studies with *S. aureus* V8 protease digests showed that although the intensity ratios of the immunoreactive peptides varied between the isozymes, no peptide(s) unique to isozyme 1 (Fig. 7), 2 (Fig. 8) or 4 was detected.

d) Determination of the basis of the charge heterogeneity: The charge differences observed in GAA may be due to differences in amino acid composition or modification of the oligosaccharide side chains. To distinguish between these two possibilities, the chromatofocusing-purified electrophoretic forms of GAA isozymes 1, 2 and 4 were treated with *V. cholerae* neuraminidase, acid and alkaline phosphatase and N-glycanase. The latter enzyme hydrolyzes N-asparagine-linked oligosaccharides from glycoproteins and glycopeptides. None of the GAA isozymes contained sialic acid residues, since neuraminidase treatment had no effect on their respective pIs. Removal of the oligosaccharide side chain by N-glycanase demonstrated that both subunits of isozymes 1, 2 and 4 were reduced in size. As shown in Fig. 19, N-glycanase treatment of the isozymes resulted in an ∿ 3 kDa decrease in molecular weight in each of the isozyme subunits. Several new low molecular weight bands were occassionally detected following N-glycanase treatment, presumably due to partial cleavage. That the proteins were totally deglycosylated was shown by the failure of the treated enzymes to bind fluoresceinated Concanavalin A. Attempts to locate the deglycosylated protein in an IEF

Figure 19: N-Glycanase digestion of GAA isozymes 1, 2 and 4. Proteins were electrophoresed in a 10% SDS-polyacrylamide gel, immunoblotted and stained with MC Ab Sp2/53. Lanes 1, 3 and 5, undigested isozymes 4, 2 and 1, respectively; lanes 2, 4 and 6, N-glycanase-digested isozymes 4, 2 and 1, respectively. Each lane contains 0.25 µg enzyme protein.


73 K
67 K
1
2
3
4
5
6

system have been unsuccessful. However, the treated protein may focus outside the pH range of 3.5 to 10. Further studies to determine the charge of the deglycosylated proteins are indicated.

Subsequent studies were performed to determine whether or not the electrophoretic forms of GAA are due to phosphorylation of the oligosascharide side chains. The isozymes were treated with acid and alkaline phosphatase and electrophoresed on IEF gels. No change in the number of electrophoretic forms or in their respective pIs was detected, indicating that the mature enzyme forms contain little or no phosphate groups.

F. Comparative Peptide Mapping of GAA Subunits:

If, indeed, the charge heterogeneity of the GAA enzyme forms is due to differences in the protein backbone of the molecules, these differences could result in the generation of unique peptides upon protease digestion. Furthermore, isozyme 2, which has a characteristically reduced catalytic activity toward glycogen, must have a major structural alteration at or near the active center for glycogen, presumably due to an amino acid substitution. Cleavage of these enzyme forms with proteases should yield unique peptide(s) which may be identified by reserved phase HPLC. Isolation and sequencing of such a peptide(s) would allow the definition, at the protein level, of the amino acid substitution responsible for the abnormal kinetic and catalytic behavior of this isozyme. Additionally, most purified GAA preparations, which on SDS-PAGE show two major subunits (67 and 73 kDa), show several "contaminating" bands. Of these, the most common are a high molecular weight protein with a $M_r \sim 96$ kDa and a low molecular weight form with a

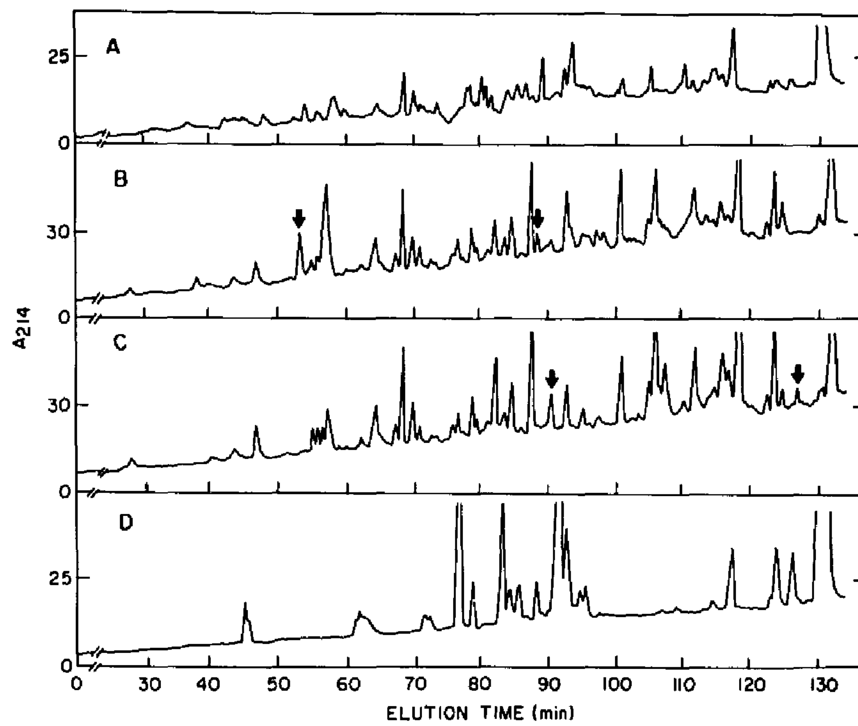
$M_r \sim$ 20 kDa. All forms are glycoproteins, as demonstrated by their ability to bind fluoresceinated conconavalin A. Peptide profiles from these protein forms should determine their relatedness to the two major subunits of GAA.

Studies were therefore conducted to a) isolate to homogeneity, the "precursor" (96 kDa), the two major subunits (67 and 73 kDa), and the 20 kDa protein; b) establish conditions for obtaining proteolytic digests of consistent quality, and c) develop HPLC methodologies to obtain optimal separation of the peptides generated.

Electroelution from preparative SDS-gels was essentially as described by Hunkapillar et al. (54). The eluted proteins were concentrated on a Centricon 10 microconcentrator (Amicon Corp., Danvers, MA). Trypsin digestion of homogeneous electroeluted GAA was performed as described. The resultant peptides were then resolved by chromatography on a 5 μ Vydac C_4 reversed phase column (4.6 mm i.d. x 25 cm), which had been equilibrated with 100% water containing 0.05% trifluoroacetic acid. Sequential linear gradients of 0-60% acetonitrile (80% acetonitrite, 20% water with 0.05% trifluoroacetic acid) from 0-180 min and 60-80% acetonitrile from 180-240 min were applied and the column was developed at a flow-rate of 0.7 ml/min. The eluate was monitored by adsorbance at 214 and 280 nm.

The peptide profiles obtained with the trypsin-digested electroeluted proteins from a typical GAA isozyme 1 preparation are shown in Fig. 20. The pattern obtained with the 73 kDa subunit (Panel B) and the 67 kDa subunit (Panel C) are virtually identical. However, several peptides unique to each subunit (arrows) were consistently observed. Panel A shows the peptide profile of the 96 kDa protein, which clearly

Figure 20: Tryptic peptide maps of human placental GAA. The enzyme forms were purified by electroelution and digested with TPCK-trypsin. Peptides were resolved by reversed phase HPLC. Panel A, 96 kDa precursor; Panel B, 73 kDa subunit; Panel C, 67 kDa subunit; and Panel D, 20 kDa glycoprotein co-purified with GAA.


A
B
C
D
25
0
30
0
30
0
25
0
A214
0
30
40
50
60
70
80
90
100
110
120
130
ELUTION TIME (min)

demonstrates the relatedness of this protein to the 67 and 73 kDa species. Major differences in the patterns occur between elution time 75 to 93 min and 121 to 128 min. It is unclear at this time, whether these differences in the peptide profiles are due to incomplete proteolysis, or whether they truly reflect differences in the protein backbone. Nevertheless, the relatedness of this large MW protein to the mature processed forms of M_r 67 and 73 kDa have been established.

Panel D shows the peptide profile obtained with tryptic digests of the 20 kDa protein which consistently co-purified with GAA. Clearly, this protein shows little, if any relationship to any of the GAA enzyme forms, and therefore most likely is a co-purified contaminant.

The peptide profile of isozyme 4 was compared to that of several of the electrophoretic forms of isozyme 1. The preparations used for these studies were purified by chromatofocusing, but were not electroeluted from SDS-gels. Thus, the preparations contained varying proportions of both the 67 and the 73 kDa subunits (isozyme 4: predominantly 73 kDa form; isozyme 1: predominantly 67 kDa form). The proteolytic peptide patterns were indistinguishable. The tryptic digests of isozyme 1 and 4 were also subjected to analytical SDS-PAGE, Western blotted and immunostained with PC Abs and MC Ab Sp2/53. No peptides unique to a particular isozyme were observed. Therefore, with the exception of a different pI, isozymes 1 and 4 appear to be biochemically, physically and immunologically identical.

G. Determination of the Amino Acid Composition and N-Terminal Sequences of GAA Isozyme 1:

The electroeluted subunits of GAA described above were analyzed for their amino acid composition and N-terminal sequence. The amino acid compositions of the 67 and 73 kDa subunits were very similar (See Table 6). Both subunits had a very high leucine content. Three amino acid residues (Thr, Gln/Glu and Pro) were noticably higher in the 73 kDa subunit. Additionally, approximately 33% of the amino acid residues in each subunit were hydrophobic. Table 6 also shows the amino acid composition of the bovine liver GAA previously reported by Bruni et al. (65). With the exception of a higher proline and leucine content in the human enzyme, the composition of the bovine enzyme is virtually identical.

The unblocked, mature 73 kDa subunit provided an amino terminal sequence of 16 residues (see Fig. 21). Of the residues identified, 50% were encoded by one or two codons. Such a high percentage of low redundency codons will greatly reduce the complexity of the oligonucleotide mixtures that must be constructed to include all possible codon combinations predicted from the amino-terminal amino acid sequence. These oligonucleotide probes, as well as additional protein sequences, will be invaluable in experiments designed to obtain additional cDNA clones that include the entire prepropeptide coding region as well as a possible 5' untranslated region. These clones should allow the characteriation of the genomic structure and organization as well as the elucidation of the molecular defect(s) in unrelated patients with glycogenosis II. The 15 amino acid sequence obtained from the amino-terminus of

TABLE 6

Amino Acid Composition of Human GAA

Amino Acid Residue	Number of Residues, Integral No.		
	Human Placental GAA		Bovine Liver GAA[d]
	67 kDa[c]	73 kDa[c]	107 kDa
Asx[a]	52	55	54
Thr	38	45	49
Ser	43	45	43
Glx[b]	58	64	63
Pro	57	65	49
Gly	52	52	47
Ala	45	45	41
Val	42	43	45
Met	13	16	14
Ile	18	19	21
Leu	72	76	65
Tyr	26	28	24
Phe	37	40	33
His	20	22	12
Lys	8	11	9
Arg	32	37	28
Trp	-	-	22
Half-Cys	-	-	8

[a]Asx = Asn or Asp

[b]Glx = Gln or Glu

[c]Subunit molecular weight

[d]Ref. 65

Figure 21: N-Terminus amino acid sequence of the 67 and 73 kDa subunits of human placental GAA. A, 73 kDa subunit; B, 67 kDa subunit. Note the overlapping sequence of Pro-Ser-Tyr-Ser-Val-Ser-Phe in B. Tentative assignments are shown in parentheses and X denotes an unidentifiable amino acid residue.

A

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Met	Gln	Pro	Trp	(Thr)	Phe	Phe	Pro	Pro	Ser	Tyr	X	Pro	X	Lys	Lys

B

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Ala	Pro	Ser	(Pro)	Leu	(Tyr)	(Glu)	Val	X	Phe	(Pro)	Leu	Phe	Gly	(Val)
		(Leu)	(Tyr)	(Ser)	(Val)	(Ser)	(Phe)		(Ala)	(Ser)	Pro		Pro	
		(Val)				(Thr)	(Thr)							

the 67 kDa form was not as informative, since the identification of eight of the amino acids could not be definitively identified (see Fig. 21).

Nevertheless, these data demonstrated that the amino-terminal sequences of the two subunits were distinct. Furthermore, the results supported the concept that the 67 kDa subunit was derived from the 76 kDa form by the removal of an N-terminal peptide(s). Alternatively, the two subunits could result from differential proteolytic cleavage of the 96 kDa precursor molecule, resulting in two enzyme forms, each with unique N-terminal sequences.

Belenky and Rosenfeld (62) obtained data with GAA from human liver that suggested that the enzyme consisted of at least three structurally different subunits. Apparently homogeneous enzyme was subjected to dansylation and further acid hydrolysis to determine the N-terminal amino acid composition. Thin-layer chromatography of the acid hydrolysate of the DNS enzyme showed DNS derivatives of alanine, glycine and glutamic acid. The present study clearly demonstrated that human placental GAA contained only two subunits. In agreement with the result obtained by Belenky and Rosenfeld (62), the 67 kDa subunit was shown to have alanine as the N-terminus amino acid. The assignment of methionine as the N-terminus amino acid in the 73 kDa subunit, however, clearly differs from the results obtained in (62). This discrepancy in results is most likely due to differences in the purity of the enzyme preparations. Indeed, when the "homogeneous" GAA in (62) was analyzed by SDS-PAGE, the enzyme was resolved into three components with different molecular weights. Our own experience has shown that several lower molecular weight proteins do co-elute with GAA using standard purifi-

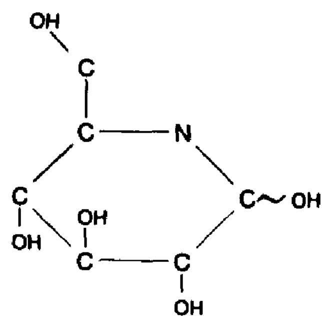
cation procedures. It is highly probable that the presence of one or more of these contaminants resulted in data that suggested the presence of three structurally different GAA subunits with the N-terminal alanine, glycine and glutamic acid, respectively.

H. Kinetic Characterization of GAA Isozymes:

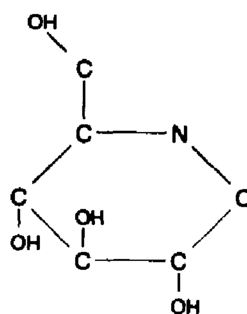
1. Use of Inhibitors to Characterize the Normal and Mutant Substrate Binding and Catalytic Site(s): Initial studies were aimed at identifying reagents which might provide information on the nature of the active center in the normal enzyme forms and which would prove informative in elucidating the alteration in isozyme 2 which results in the greatly reduced catalysis of glycogen. Potential inhibitors (including those with substituents in the pyranose ring and at selected OH-groups) were tested with the normal isozyme 1, using maltose as substrate, to determine their effectiveness and to obtain an approximate I_{50} value. Subsequently, the inhibitors were tested with isozymes 1 and 2, using both maltose and glycogen as substrates, to define the type and potency of inhibition.

As can be seen in Table 7, all of the OH-group substituted compounds were poor inhibitors of GAA. In comparison, compounds with S or, particularly, N substituents in the O position of the glucopyranose were more potent inhibitors of GAA activity. Some of the more interesting inhibitors of GAA are shown in Fig. 22. None of the inhibitors tested showed a difference in the potency of inhibition with isozymes 1 and 2 when maltose was the substrate. However, four of the inhibitors (see Table 8) showed a reproducible difference ($\geq$ 15%) in the potency of

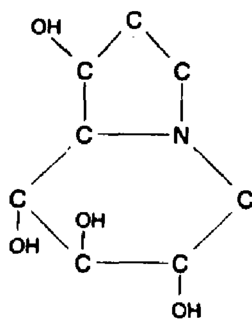
Figure 22: Pyranose ring-substituted inhibitors of human GAA.



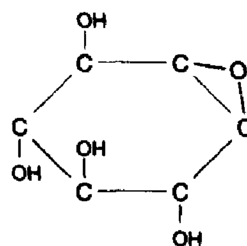
nojirimycin



1-deoxynojirimycin



castanospermine



conduritol B epoxide

TABLE 7

Inhibition of GAA

Inhibitor	I_{50}[a]	Inhibition
OH-Group Substituted Compounds		
L-glucose	18 mM	non-competitive
α-methylglucoside	50 mM	competitive
α-glucose-1-PO_4	50 mM	non-competitive
β-glucose-1-PO_4	16 mM	non-competitive
D-glucose-L-cysteine	31 mM	uncompetitive
D-glucosamine-1-PO_4	250 mM	nd[b]
D-glucosamine-6-PO_4	400 mM	nd
D-glucosamine HCl	420 mM	Competitive
N-acetylglucosamine	800 mM	nd
1-thio-β-D-glucose	750 µM	competitive/mixed
Ring-Substituted Compounds		
Nojirimycin	1.4 µM	competitive/mixed
1-deoxynojirimycin	1.1 µM	competitive/mixed
N-dodecyldeoxynojirimycin	1.0 µM	competitive/mixed
Castanospermine	8.0 µM	competitive/mixed
5-deoxy-5-thio-D-glucose	125.0 mM	nd
Conduritol B-epoxide	1.0 mM	covalent

[a] I_{50} was determined with isozyme 1 of GAA, using maltose as substrate.

[b] nd = not determined.

TABLE 8

Inhibition of GAA Isozymes 1 and 2

Inhibitor	% Inhibition[a]	
	Isozyme 1	Isozyme 2
L-Glucose	71	30[b]
α-glucose-1-PO_4	51	31[b]
B-glucose 1-PO_4	72	55[b]
D-glucose-L-cysteine	78	100[b]
D-glucosamine-1-PO_4	31	34
D-glucosamine-6-PO_4	66	72
D-glucosamine HCl	69	54
N-acetylglucosamine	59	65
Nojirimycin	80	81
Deoxynojirimycin	66	77
N-dodecyldeoxynojirimycin	20	25
5-thio-D-glucose	98	90
Castanospermine	66	63
Conduritol-B-epoxide	49	68[c]

[a]% Inhibition was determined using glycogen as substrate

[b]Inhibition varied > 15% between isozymes

[c]At higher concentrations of CBE, the difference in inhibition was not observed.

inhibition between the two isozymes when glycogen was the substrate. Since these compounds exerted non- or uncompetitive inhibition patterns (see Table 7) none of these inhibitors exerted their influence at the active center of the enzymes. Nevertheless, additional studies using these compounds may provide insight into the residues near the active center which are critical for the optimal efficiency of the enzyme forms in catalyzing glycogen.

2. CBE: A Covalent Active Site-Directed Inhibitor of GAA: CBE has been shown to be a covalent active site inhibitor of many β-glucosidases (35). Studies were therefore performed to determine whether CBE inhibited human GAA, and whether this inhibition was irreversible (i.e. covalent binding to the active center).

Preliminary experiments indicated that CBE was not a highly potent inhibitor of human GAA. When the inhibitor was tested in the presence of substrate (maltose or glycogen), very low levels of inhibition were obtained even at relatively high concentration (> 3 mM). However, when the enzyme was preincubated with CBE and then analyzed, the levels of inhibition approached 100% at concentrations of > 5 mM. Thus, the substrates maltose and glycogen protected the enzyme from inactivation by CBE, an indication that this inhibitor competes for the same binding site as the substrates.

Studies on the time course of inactivation further demonstrated that isozymes 1 and 2 were inhibited at the same rate by CBE, regardless of whether assayed against maltose or glycogen. To demonstrate that the CBE binding to GAA was indeed irreversible (i.e., covalent binding), the enzyme was preincubated with CBE under conditions which

resulted in at least 50% inactivation of the enzyme. The CBE-enzyme mixture was dialyzed for 24 h against 4,000 volumes of buffer and assayed with maltose substrate. The percentage of inhibition prior to and post-dialysis were identical.

To determine whether each subunit of the GAA molecule was capable of binding CBE, the enzyme was preincubated with ^{3}H-CBE (specific activity: 1.5 x 10^{8} cpm/ µmole) at a molar ratio of 25,000:1 (CBE:GAA). This ratio resulted in a > 90% inactivation of the enzyme following a five day incubation at 25°C. The CBE-labeled enzyme was subjected to SDS-PAGE, Western blotted and immunostained with MCAb Sp2/53 in order to locate the 67 and 73 kDa GAA subunits. The nitrocellulose was cut into strips, treated overnight with Protosol, scintillation fluid was added, and the vials were counted 24 h later in a liquid scintillation counter. Counts were detected only in strips containing the GAA subunits and were distributed nearly equally between the 67 and the 73 kDa subunits.

These results indicate that the covalent, active site-directed inhibitor CBE may be of great value in experiments designed to identify and isolate proteolytic peptides containing the active center. Since several of the mutant enzymes studied (catalytically active and inactive) have retained the ability to bind substrates, and therefore, should bind CBE, this reagent may be of great use in isolating normal and mutant active site containing peptides for sequencing and immunochemical studies.

VI. CONCLUSIONS

The overall objective of this proposal was to investigate the nature of the observed clinical and biochemical heterogeneity in glycogenosis II. Accomplishment of this goal was dependent on the development of procedures and reagents which would allow not only the accurate identification of the GAA isozymes and their electrophoretic forms, but also the detailed analysis of the structure and function of enzyme forms.

An IEF system was developed which resulted in the unequivocal identification of the three major GAA isozymes (types 1, 2 and 4), as well as the resolution of their multiple electrophoretic forms. This system was used to screen 214 placentae to determine their isozyme pattern and calculate allelic frequencies. Based on the sampling of the New York population, the gene frequencies were calculated to be 0.942, 0.017 and 0.041 for alleles 1, 2 and 4, respectively. These values are somewhat different from those obtained in a Canadian survey (20) and may be due to ethnic differences in the population sampled.

All of the isozymes and their electrophoretic forms were subsequently purified using conventional chromatographic and chromatofocusing procedures. Isozymes 1 and 2 were each resolved into four electrophoretic species. Each of the chromatofocusing-isolated forms had the respective immunologic, physical and kinetic properties of the conventionally purified isozymes. Of further interest was the finding that as the electronegativity of the proteins increased, the subunit composition changed from predominantly 73 kDa to predominantly 67 kDa subunits. Thus, isozyme 4, which is the most electropositive of the isozymes, consists almost entirely of a 73 kDa subunit, whereas the most electro-

negative form (isozyme 1) demonstrates only a 67 kDa subunit. This shift in molecular weight is most likely due to proteolytic cleavage of the enzyme. Indeed, evidence has been presented which indicates that the final stages of maturation of the GAA protein occur in the lysosome and are proteolytic in nature (25).

Tryptic peptide profiles of the 67 and 73 kDa subunits resolved by reversed phase HPLC showed that the subunits are virtually identical in composition. Several peptides unique to each subunit were consistently observed, but whether these differences are sufficient to account for the change in size and charge remains to be determined. That the charge heterogeneity is due to changes in the protein backbone of the molecule and not due to modifications on the oligosaccharide side chain(s) is suggested by the finding that the mature GAA contains no sialic acid and little or no phosphate.

Tryptic peptide profiles of the 96 kDa precursor of GAA also were obtained. Comparison of these profiles with those of the 67 and 73 kDa mature enzyme forms clearly demonstrate the relatedness of these proteins. The amino-terminal sequences of the two subunits were distinct, suggesting that the 67 kDa subunit was derived from the 73 kDa form by the removal of an N-terminal peptide(s). Alternatively, the two subunits could result from the differential proteolytic cleavage of the 96 kDa precursor molecule, resulting in two enzyme forms, each with a unique N-terminus. Whether the proteolytic processing of GAA takes place in a sequential fashion or from simultaneous differential cleavage of the precursor remains to be determined. However, the finding of a number of intermediates between 96 kDa and 67 kDa is suggestive of a sequential mechanism.

Immunologic studies, using PC and newly developed MC Abs, resulted in the discrimination among and between the subtypes of glycogenosis II and their variants. Of particular interest was the identification of a mutant protein in the unique CRM-positive infantile subtype. This protein was catalytically inactive, had reduced immunoreactivity with PC Abs, was pH-labile, and had an abnormal pI. Furthermore, MC Ab Sp2/53, which recognizes an epitope at or near the catalytic center of the normal enzyme, cross-reacted with three proteins in subtypes previously defined as CRM-negative using PC Abs. The availability of active site-directed MC Abs, and the identification of a covalent active site-directed inhibitor of GAA (CBE) should allow the isolation of peptides containing the substrate binding and/or the catalytic site from normal and mutant enzyme forms. Immunologic, biochemical and molecular studies of these isolated enzymes and peptides should result in the correlation of specific molecular lesions with abnormal physical and kinetic properties of the enzymes and will ultimately identify structure-function relationships critical to normal GAA functions.

Furthermore, amino acid sequencing analysis will identify the exact residues necessary for optimal enzyme activity as well as define mutations which result in reduced, altered, or complete absence of enzyme activity. The identification of the specific base substitutions in GAA from selected subtypes and/or variant patients will allow the synthesis of mutation-specific oligonucleotide probes which can be used for accurate diagnosis of carriers and affected individuals, as well as for prenatal detection of the specific disease subtypes and/or variants.

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