STRUCTURAL AND FUNCTIONAL ANALYSIS OF LYSOSOMAL α-GLUCOSIDASE IN RELATION TO GLYCOGEN STORAGE DISEASE TYPE II

STRUCTURELE EN FUNCTIONELE ANALYSE
VAN HET LYSOSOMALE α-GLUCOSIDASE IN RELATIE TOT
GLYCOGEEN STAPELINGSZIEKTE TYPE II

PROEFSCHRIFT

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Gutta cavat lapidem, non vi, sed saepe cadendo

(Ovidius)

Voor pap en mam

Aan Gerard

CONTENTS

The one and three letter code of amino acids

amino acid	three letter code	one letter code
alanine	Ala	A
arginine	Arg	R
asparagine	Asn	N
aspartic acid	Asp	D
cystine	Cys	C
glutamine	Gln	Q
glutamic acid	Glu	E
glycine	Gly	G
histidine	His	H
isoleucine	Ile	I
leucine	Leu	L
lysine	Lys	K
methionine	Met	M
phenylalanine	Phe	F
proline	Pro	P
serine	Ser	S
threonine	Thr	T
tryptophan	Trp	W
tyrosine	Tyr	Y
valine	Val	V

OBJECTIVES

Glycogen storage disease type II is caused by the inherited deficiency of lysosomal α-glucosidase. The enzyme belongs to the class of α-glucanases and hydrolyses lysosomal glycogen to glucose.

The aim of the work described in this thesis was to provide insight in the structure-function relationship of lysosomal α-glucosidase and to elucidate the mutations underlying the lysosomal α-glucosidase deficiency of patients with various clinical forms of glycogen storage disease type II.

CHAPTER 1

α-GLUCANASES

α-GLUCANASES

Introduction

α-Glucanases is a collective name for enzymes that are capable to hydrolyse α-glucosidic linkages in glucose polymers such as glycogen and starch and in a variety of other oligo- and polysaccharides. Glycogen is a polysaccharide entirely composed of glucose molecules. It is the most abundant and widespread form of glucose and the most important store of energy in the animal cell. Also yeast, fungi and bacteria store energy in the form of glycogen (Huijing, 1975). The glucose residues in glycogen are joined by α-1,4 and α-1,6 linkages, which results in a highly branched structure (Figure 1). The α-1,6 linkages create the branching points. Approximately 93% of the glucosidic linkages are of the 1,4 type and 7% are of the 1,6 type.

Starch is the nutrient reservoir of plants and is also a homopolymer of glucose. Starch is made up of two components, amylose and amylopectin. Amylose is a mainly linear polysaccharide composed of glucose molecules in α-1,4 linkage and contains only few α-1,6 branching points. Amylopectin, the branched form of starch, contains about

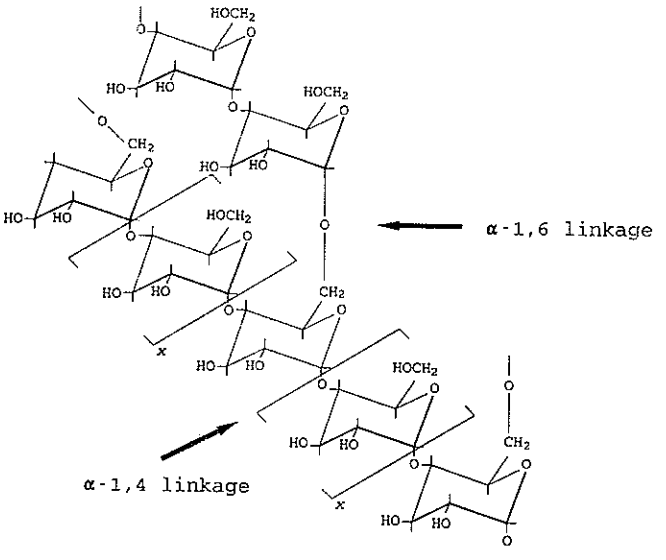

Figure 1: Molecular structure of glycogen. *x:* the number of α-1,4 linked glucose molecules is approximately thirteen.

3% of α-1,6 glucosidic linkages. Some plants such as sweet corn store their energy not only in starch but also in glycogen (Huijing, 1975).

The α-glucanases are subdivided in different classes, such as α-amylases, glucoamylases, α-glucosidases and cyclomaltodextrin glucanotransferases. Several of the α-glucanases have been isolated and characterized, and the corresponding genes have been cloned. This chapter describes the proposed mode of action of these enzymes, their substrate specificities and their structural and functional homologies.

Hydrolysis of the α-glucosidic bond

Hydrolysis of the α-glucosidic bond by α-glucanases is believed to occur mechanistically in a similar way as the cleavage of peptide bonds by lysozyme and involves a "catalytic acid" (general acid) and a "catalytic base" (general base) (Figure 2) (Vernon, 1967). The catalytic acid donates a proton to the glucosidic oxygen atom between two glucose molecules (Figure 2B). This protonated complex is very unstable and falls apart in a positively charged carbo-cation and a neutral sugar residue (Figure 2C). The carbo-cation is stabilized by the catalytic base. A water molecule supplies subsequently a hydroxyl group (OH^-) to the carbo-cation and a proton (H^+) to the deprotonated catalytic acid of the enzyme (Figure 2D and 2E). This way the catalytic cycle is completed.

α-Amylases

α-Amylases (1,4-α-D-glucan glucanohydrolase EC 3.2.1.1) are widely distributed in mammals, plants, and microorganisms. They are endo-enzymes and catalyse the hydrolysis of α-1,4 glucosidic linkages of starch, glycogen and related oligo- and polysaccharides. They can bypass the α-1,6 branch points in α-glucans. The reaction products of α-amylases are liberated in the α-configuration as α-maltose, α-glucose and α-limit dextrins. The specificity of α-amylases varies with their source, and each α-amylase produces a characteristic collection of oligosaccharides upon hydrolysis of a glucan. The specificity of α-amylases for the α-1,4 glucosidic bond is not absolute. Some α-amylases are able to hydrolyse α-1,6-glucosidic bonds in addition, but the rates of hydrolysis are much lower than for α-1,4 bonds (Sakano *et al.*, 1985). α-Amylases are metalloenzymes and bind 1 mole of calcium per mole of enzyme (Thoma *et al.*, 1971).

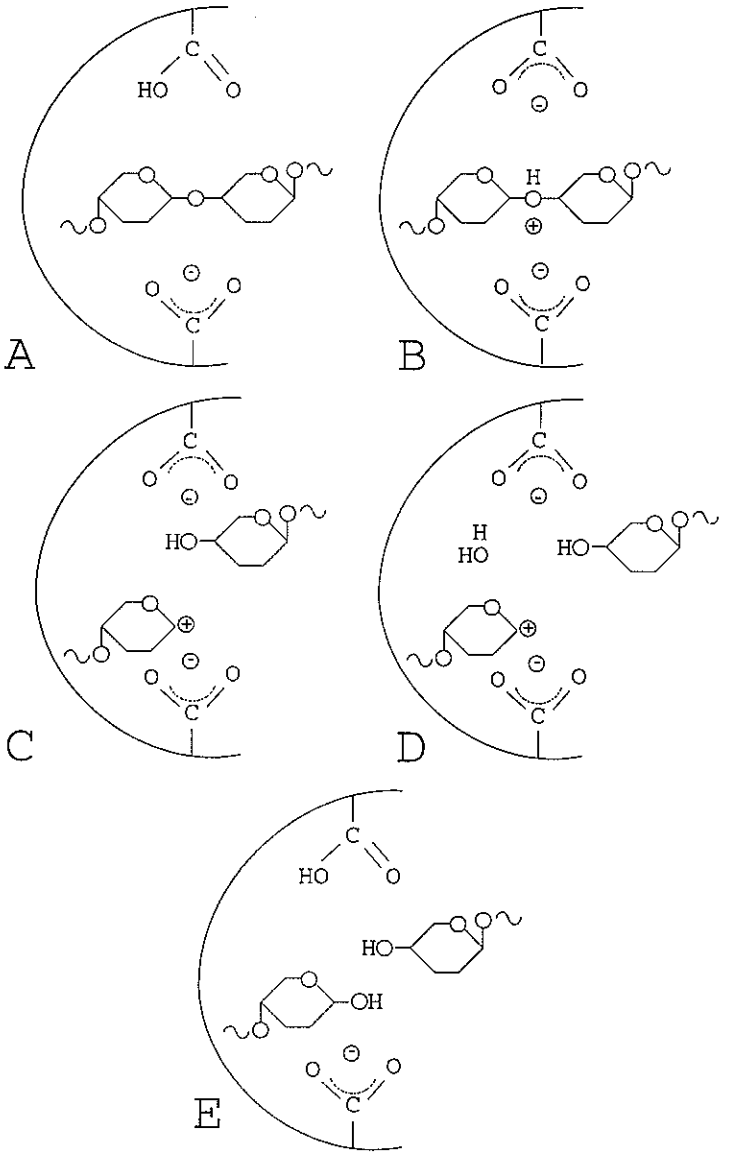

Figure 2: Schematic representation of the enzymatic hydrolysis of α-glucans.
The glucose moieties are joined to each other via an α-glucosidic linkage. CO_2H is the catalytic acid and CO_2^- the catalytic base.

The enzymes are important tools in starch industry and are used for instance in the production of beer and pharmaceuticals (Vihinen and Mäntsälä, 1989).

Most α-amylases are secretory proteins. They have an amino terminal signal peptide when synthesized but this signal is lost before secretion. Intracellular α-amylases do occur in some microorganisms, but there is uncertainty with respect to their function since starch is neither synthesized nor is taken up by these microorganisms (Walker, 1965; Narayanan and Shibuya *et al.*, 1967; Kato *et al.*, 1975; Champs *et al.*, 1983; Srivastava, 1984; Shibuya *et al.*, 1986).

A large number of α-amylase genes from different species has been cloned. Although the genes from closely related species are quite similar (e.g. the genes of *Bacillus stearothermophilus* and *Bacillus licheniformis*, or of *Xanthomonas campestris* pv. *campestris* and *Aeromonas hydrophila* (Gray *et al.*, 1986; Hu *et al.*, 1992)), the sequence of α-amylases from mammals, plants and microorganisms differ markedly. However, when the amino acid sequences of the α-amylases from widely different species were aligned, there were at least four short regions of homology found (Friedberg, 1983; Rogers, 1985; Ihara *et al.*, 1985; Nakajima *et al.*, 1986; MacGregor, 1988; Svensson, 1988; MacGregor & Svensson, 1989; Itkor *et al.*, 1990; Schneider *et al.*, 1992). These regions have been proposed to be essential for the function of α-amylases because they are spaced at similar intervals along the proteins. They presumably form the active site, the substrate-binding site and the site for binding the stabilizing calcium ion. As these regions also occur in several other α-glucan cleaving enzymes they will be discussed in more detail at the end of this chapter.

To obtain more information on the mode of action of α-amylases, the structures of Taka-amylase A from *Aspergillus oryzae* (TAA), of acid α-amylase from *Aspergillus niger* and of α-amylase from porcine pancreas (PIG) were investigated by X-ray crystallography (Matsuura *et al.*, 1984; Buisson *et al.*, 1987; Boel *et al.*, 1990). The folding of the three molecules is essentially the same. The enzymes contain a N-terminal $(\beta/\alpha)_8$-barrel catalytic domain (domain A), which is similar in structure to that of triose phosphate isomerase (Banner *et al.*, 1975) and of several other enzymes with very different functions (reviewed in Farber and Petsko, 1990). A $(\beta/\alpha)_8$-barrel is a structure that contains 8 parallel β-strands surrounded by 8 α-helices. Each inner β-strand is connected to an outer α-helix (Figure 3). In α-amylases, a smaller domain (domain B) is

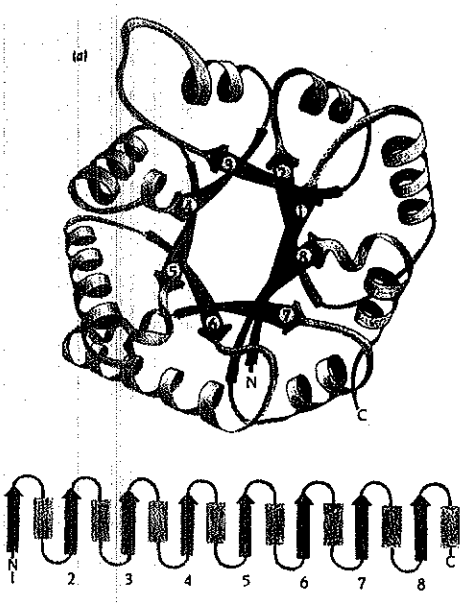

Figure 3: Schematic representation of the $(\beta/\alpha)_8$-barrel structure. The ß-strands are numbered.

inserted between the third β-strand and the third helix of the $(\beta/\alpha)_8$-barrel. This domain is thought to participate in the binding of substrate. The C-terminal region of α-amylases forms domain C, in which the protein chain is folded into an 8-stranded antiparallel β-barrel. The order of the β-strands corresponds to the so-called Greek key topology. The active site of some α-amylases was identified by studying the structural difference between plain crystals and crystals formed in the presence of a substrate-analogue (Payan *et al.*, 1980; Matsuura *et al.*, 1984). The active site corresponds to a cleft located between domain A and B. However, different pairs of acidic residues were proposed to be the catalytic residues in TAA and PIG: Glu-230 and Asp-297 in TAA and Asp-197 and Asp-300 in PIG (corresponding to Asp-206 and Asp-297 in TAA) (Table 1). The three corresponding acidic residues in *Bacillus subtilis* α-amylase (Asp-176, Glu-208 and Asp-269), were mutated to their amide forms to study their role in α-glucan catalysis (Takase *et al.*, 1992). Mutation of each of the three residues was shown to lead to a complete loss of catalytic activity while significant starch-binding activity was retained. This points to a critical role of all three residues for the catalytic reaction, but does not assign the catalytic acid and base definitely. More information was obtained by studying the affinity of these catalytic site mutants for the pseudo-oligosaccharide inhibitor acarbose. The affinity was

Table 1: Carboxylates proposed to be catalytic residues.

ENZYME	SOURCE	CARBOXYLATES		
α-amylase	Aspergillus oryzae (TAA)	(Asp-206)	Glu-230	Asp-297
α-amylase	Pig	Asp-197	(Glu-233)	Asp-300
α-amylase	Bacillus subtilis	Asp-176	Glu-208	Asp-269
α-amylase	Aspergillus niger	Asp-206	Glu-230	(Asp-297)
CGT ase	Bacillus sp. #1011	Asp-229	Glu-257	Asp-328

greatly reduced by the Asp-269 → Asn and the Glu-208 → Gln substitutions but less so by the Asp-176 → Asn substitution. Therefore Takase concluded that Glu-208 and Asp-269 were the key catalytic residues in accordance with the proposal of Glu-230 and Asp-297 made for TAA (Takase, 1992). A number of amino acid residues with charged as well as aromatic and non-polar residues seem to participate in substrate binding via hydrogen bonds and hydrophobic interactions (Buisson *et al.*, 1987).

From the crystal structure of PIG four amino acid residues have been proposed to be involved in Ca^{2+} binding (Buisson *et al.*, 1987). Two of these residues come from domain A (Asn-100 and His-201) and two from domain B (Asp-159 and Asp-167). By comparing the TAA and the PIG structures, Buisson *et al.* (1987) concluded that the Ca^{2+} binding sites are similar in both proteins. The corresponding positions in TAA are Asn-121, Asp-163, Asp-175 and His-210. In contrast to these conclusions, Boel *et al.*, (1990) have proposed that eight ligands bind Ca^{2+} in both the *Aspergillus* α-amylases: Asp-175 in a bidentate mode, Asn-121, Glu-162, Glu-210 in *A. niger* / His-210 in TAA, and three water molecules. They conclude that Asp-163 was assigned incorrectly by Buisson *et al.*, (1987). The ligands of the Ca^{2+} ion belong to domains A and B. As the active site cleft is located between these two domains, it is suggested that the essential Ca^{2+} ion stabilises the cleft by inducing an ionic bridge between domains A and B (Buisson *et al.*, 1987). Boel *et al.*, (1990) have identified a second Ca^{2+} binding site in acid α-amylase from *A. niger*. It involves residues presumed to play a catalytic role (Asp-206 and Glu-230) (see Table 1). This explains why Ca^{2+} at higher concentrations has an inhibitory effect on α-amylase action.

MacGregor (1988) took the crystal structure of TAA as a model and tried to gain insight in the structural similarities and differences between α-amylases of bacteria, fungi, plants and mammals. To this end she compared the amino acid sequences of the α-amylases and used computer programs to predict the secondary structure. It became evident that all α-amylases had the same basic structure, the $(\beta/\alpha)_8$-barrel. The amino acids forming the loops that connect the different β-sheets and α-helices of the barrel vary widely in length and in composition. These variations are believed to account for the known variations in action pattern of the α-amylases of different species (MacGregor, 1988).

β-Amylases

β-Amylases (1,4-α-D-glucan maltohydrolase EC 3.2.1.2) are common in plants, but only few microorganisms contain these enzymes. β-Amylase is an exoenzyme that hydrolyses the penultimate α-1,4 bond of starch, glycogen and related oligo- and polysaccharides. β-Maltose is produced by inversion of the anomeric configuration of the liberated maltose. The enzyme cannot bypass α-1,6 glucosidic bonds, but there is no restriction on the hydrolysis of linear chains (Marshall, 1974).

β-Amylases are extracellular enzymes like the α-amylases. Crystallographic data on soybean β-amylase have revealed a $(\beta/\alpha)_8$-barrel structure, different from that of α-amylases. The β-amylase type of barrel is predicted to exist also in the β-amylases from other higher plants as well as from *Bacillus polymyxa* (Friedberg and Rhodes, 1988). The amylase gene product of this latter microorganism is unusual in that it contains in tandem both an α- and a β-amylase (Uozumi *et al.*, 1989). The α-amylase has the characteristic A- B- and C-domains (Jespersen *et al.*, 1991).

Exo-1,4-α-D-glucosidases

Glucoamylase, amyloglucosidase, γ-amylase and lysosomal α-glucosidase (1,4-α-D-glucan glucohydrolase EC 3.2.1.3) are exo-hydrolases acting on both α-1,4 and α-1,6 linkages. Some split also α-1,3 glucosidic bonds. These enzymes have a higher specificity for polysaccharides than for oligosaccharides. β-Anomeric glucose residues are released as reaction product.

Glucoamylases are widely distributed from eukaryotic micro-organisms to

vertebrates, but they are rare in bacteria (Sorensen *et al.*, 1982; Manjnath *et al.*, 1983; Yamashita *et al.*, 1985; Pretorius *et al.*, 1991). Fungal glucoamylases are commercially important for the degradation of starch, in the production of high-glucose syrups and ethanol (Norman, 1979). Several fungal and yeast, but only one bacterial glucoamylase have been cloned (Boel *et al.*, 1984; Nunberg *et al.*, 1984; Yamashita *et al.*, 1985, 1987; Ashikari *et al.*, 1986; Itoh *et al.*, 1987; Hata *et al.*, 1991; Ohnishi *et al.*, 1992).

All but one of the fungal glucoamylases are 30 - 40% homologous and have four conserved regions (Itoh *et al.*, 1987). Three of them are different from those found in α-amylases. In *Aspergillus niger* glucoamylase Svensson *et al.*, (1990) identified three acidic residues (Asp-176, Glu-179 and Glu-180) which were completely protected by an inhibitor and therefore assumed to participate in the formation of the catalytic site. These three amino acid residues are shared by the other fungal glucoamylases. To determine their catalytic function, these amino acids were converted via site directed mutagenesis into Asn-176, Gln-179 and Gln-180 (Sierks *et al.*, 1990). Kinetic analysis of the mutant enzymes indicated that Glu-179 was the catalytic acid and Asp-176 the catalytic base. The negative charge of Glu-180 contributes to the high pKa value of Glu-179. This region of the glucoamylases is highly homologous with one of the four homologous regions identified in α-amylases (region II) (Svensson, 1988).

Glucoamylase from the yeast *Schwanniomyces occidentalis* showed hardly any amino acid similarity with the glucoamylases discussed above (Dohmen *et al.*, 1990). This finding was unexpected since all other glucoamylases cloned until then had a significant homology (Itoh *et al.*, 1987, Ohnishi *et al.*, 1992). Surprisingly this *S. occidentalis* glucoamylase appeared to be genetically and structurally related to human lysosomal α-glucosidase (which is also an 1,4-α-D-glucan glucohydrolase), to rabbit and human sucrase and isomaltase (intestinal brush border enzymes, EC 3.2.1.48 and EC 3.2.1.10, respectively) and to an α-glucosidase (EC 3.2.1.20) from the yeast *Candida tsukubaensis* (Hunziker *et al.*, 1986; Hoefsloot *et al.*, 1988; Dohmen *et al.*, 1990; Naim *et al.*, 1991; Kinsella *et al.*, 1991; Chantret *et al.*, 1992). A sequence identity of minimal 34% is obtained when the primary sequence of these enzymes are optimally aligned. In view of this structural similarity it is assumed that all these enzymes have evolved from the same ancestral gene, whereby the gene coding for the common precursor of sucrase and isomaltase has arisen by gene duplication (Hoefsloot *et al.*, 1988; Semenza, 1989;

Naim *et al.*, 1991; Kinsella *et al.*, 1991; Chantret *et al.*, 1992). This ancestral gene probably has coded for a protein with multiple substrate specificities, like the α-glucosidase from *C. tsukubaensis* which is able to hydrolyse α-1,2, α-1,3, α-1,4 and α-1,6 linkages. Evolutionary changes in the primary structure have ultimately led to the distinct substrate specificities. By affinity labelling and site directed mutagenesis the same aspartic acid residues in homologous positions have been identified as the catalytic bases, Asp-505 in rabbit isomaltase, Asp-1396 in rabbit sucrase and Asp-518 in lysosomal α-glucosidase (Quaroni and Semenza, 1976; Hunziker *et al.*, 1986; Hermans *et al.*, 1991). This aspartic acid residue is conserved in all these evolutionary related α-glucosidases as are eight other adjacent amino acids (Table 2). The region is homologous to the second conserved region of the other glucoamylases mentioned above.

Not much is known about the tertiary structure of glucoamylases. The existence of an α-amylase like A domain formed by a $(\beta/\alpha)_8$-barrel is not predicted for the glucoamylases (Jespersen *et al.*, 1991). Glucoamylase from *A. niger* contains two domains, a catalytic domain (residues 1-470) and a C-terminal domain (residues 509-616). The latter domain is involved in the binding of starch and has sequence homology with some other starch binding proteins (Svensson, 1988; Svensson *et al.*, 1989). Specific features of this domain will be discussed at the end of this chapter. The N-terminal and

Table 2: Conserved amino acid sequence around the proposed catalytic base of evolutionary related α-glucanases.

ENZYME	SOURCE	POS[1]	
α-glucosidase	C.tsukubaensis	521	SGIWLD**M**N**E**PSSFVIG[2]
glucoamylase	S. occidentalis	465	DGLWAD**M**N**E**VSSFCVG
isomaltase	rabbit	500	DGLWID**M**N**E**VSSFVQG
sucrase	rabbit	1389	DGLWID**M**N**E**PSSFVQG
isomaltase	human	500	DGLWID**M**N**E**VSSFIQG
sucrase	human	1389	DGLWID**M**N**E**PSSFVNG
lysosomal α-glucosidase	human	513	DGMWID**M**N**E**PSNFIRG

[1]The figures refer to the position of the first amino acid of the sequence. [2]The conserved amino acid residues are printed in bold.

C-terminal domains are separated by a region (residues 471-508) which is rich in short O-linked oligosaccharides (Gunnarsson *et al.*, 1984; Evans *et al.*, 1990). The major function of this region is to provide an extended peptide backbone and hence to create an appropriate distance between the catalytic and the substrate binding domain (Williamson *et al.*, 1992).

α-D-glucosidases

α-Glucosidases or maltases (α-D-glucoside glucohydrolase EC 3.2.1.20) hydrolyse terminal 1,4-linked α-D-glucose residues from oligosaccharides with the release of α-glucose. They act more slowly on polysaccharides.

The primary structure of the maltase gene of *Saccharomyces carlsbergensis* has been determined (Hong and Marmur, 1986). The enzyme contains the same four regions that are conserved among the α-amylases (Svensson, 1988). Alignment of the amino acid sequences indicates that *S. carlsbergensis* maltase contains a similar $(\beta/\alpha)_8$-barrel supersecondary structure as the α-amylases (MacGregor and Svensson, 1989; Jespersen *et al.*, 1991).

Cyclomaltodextrin glucanotransferases

Cyclodextrin glucanotransferases (CGTases) (1,4-α-D-glucan 4-α-D-(1,4-α-D-glucano)-transferase (cyclizing) EC 2.4.1.19) are capable to cleave α-1,4-glucosidic bonds in amylose and starch. CGTases degrade the substrates mainly to cyclodextrins, in which six to eight glucose units are joined by means of α-1,4-glucosidic bonds (Matsuzaki *et al.*, 1974; Kobayashi *et al.*, 1978). Thus, α-1,4-glucosidic bonds can be reformed through the transferase activity of CGTases, in addition to being cleaved by its amylase activity. Interestingly, lysosomal α-glucosidase, the enzyme studied in the experimental part of this thesis, also exhibits intrinsic transferase activity, since it can catalyse the transfer of glucose from maltose to polysaccharides (Palmer, 1971). CGTases have been found only in bacteria and they are all extracellular enzymes (Vihinen and Mäntsälä, 1989). They depend on Ca^{2+} ions for stability and activity (Bender, 1977).

CGTases from several species have been cloned (Binder *et al.*, 1986; Takano *et al.*, 1986; Kimura *et al.*, 1987; Sakai *et al.*, 1987; Nitschke *et al.*, 1990). The sequences

have an overall amino acid homology of about 30%. The four conserved regions of the α-amylases are also found in CGTases, with a similar spacing (Binder *et al.*, 1986; Kimura *et al.*, 1987).

The three-dimensional structure of CGTase from *Bacillus circulans* has been studied in great detail (Hoffmann *et al.*, 1989; Klein and Schulz, 1991). Five domains can be identified. The chain fold of the three N-terminal domains encompassing 492 residues resembles closely the known structure of α-amylases. Domain A forms a $(\beta/\alpha)_8$-barrel with domain B inserted after the third β-strand. Domain C folds as a β-sheet with Greek key topology. Domain D has an immunoglobin fold and is far away from the active centre. The C-terminal 100 residues form domain E, which is proposed to be involved in the binding of starch (Svensson, 1988; Svensson *et al.*, 1989). The relative positions of domains A, B and C are similar in α-amylases and CGTases, suggesting hence, that the CGTases may be considered as extended α-amylases (Hoffmann *et al.*, 1989). Even the residues involved in the Ca^{2+} ion binding are at corresponding positions in CGTases and α-amylases (Klein and Schulz, 1991).

Comparison of the amino acid sequences of CGTases and α-amylases revealed in addition that the two aspartic acid residues and the one glutamic acid residue, considered to be the catalytic residues of α-amylases, were conserved in CGTases (Table 1). These three residues in CGTase from *Bacillus sp # 1011* were substituted by the Asn or Gln analogues to analyze their function. It was shown that mutants containing Asn-229, Gln-257 or Asn-328 lost both their starch-degrading and cyclodextrin-forming activities completely, but retained their ability to bind starch. These results suggest that all three residues are essential for the reaction catalysed by CGTase (Nakamura *et al.*, 1992).

Other glucanases

The genes of a number of other hydrolases, which can split O-glucosidic linkages in starch, glycogen and related oligo- and polysaccharides have been cloned. Among these are the two genes coding for the oligo-1,6-glucosidases (EC. 3.2.1.10) from *Bacillus cereus* and *Bacillus thermoglucosidasius*. The proteins have 72% sequence similarity (Watanabe *et al.*, 1990, 1991) and *B. cereus* oligo-1,6-glucosidase is for 42% similar to *S. carlsbergensis* maltase (Watanabe *et al.*, 1990). Alignment of the amino acid sequences

along with secondary structure predictions make it likely that the N-terminal active site domains of these oligo-1,6-glucosidases take the α-amylase type $(\beta/\alpha)_8$-barrel super-secondary structure. Notably, the mammalian intestinal isomaltases from rabbit and human, which are also oligo-1,6-glucosidases do not have this α-amylase type $(\beta/\alpha)_8$-barrel catalytic domain (Hunziker *et al.*, 1986; Jespersen *et al.*, 1991; Chantret *et al.*, 1992).

The enzymes capable of degrading pullulan (a linear α-glucan consisting of maltotriose units joined by α-1,6 glucosidic linkages), pullulanase (EC. 3.2.1.41) and neopullulanase (not classified), have also the four highly conserved regions present in α-amylases. These regions are further found in dextranase (EC. 3.2.1.11), cyclomaltodextrinase (EC. 3.2.1.54) maltotetraohydrolase (EC. 3.2.1.60), isoamylase (EC. 3.2.1.68), maltohexaohydrolase (EC. 3.2.1.98) and branching enzyme (EC. 2.4.1.18) (Baeker *et al.*, 1986; Katsuragi *et al.*, 1987; Amemura *et al.*, 1988; Tsukamota *et al.*, 1988; Kuriki and Imanaka, 1989; Fujita *et al.*, 1989; Zhou *et al.*, 1989; Russell and Ferretti, 1990; Melasniemi *et al.*, 1990; Baba *et al.*, 1991; Podkovyrov and Zeikus, 1992). In spite of the low sequence similarity between these groups of enzymes Jespersen *et al.* (1991) predict that these enzymes also contain the typical $(\beta/\alpha)_8$-barrel domain.

The structural homology of α-glucanases

The α-glucanases that are compared are listed in Table 3. Table 4 shows the homologous regions of the α-glucan hydrolysing enzymes. The order of the regions I, II, III and IV, from the amino-terminus to the carboxyl-terminus, is the same for all enzymes listed.

In region I two amino acid residues are strictly conserved, namely the first: aspartic acid (D) and the last: histidine (H). Nothing is known about the functional significance of the Asp residue. For the α-amylases from *A. oryzae* (TAA) and pig, the invariant His has been demonstrated to be involved in the binding of substrate. In many of these enzymes also the fifth residue is conserved: Asparagine (N). This Asn is involved in the binding of the essential Ca^{2+} ion as was deduced from the crystal structures of the α-amylases from *A. oryzae, A. niger* and PIG and of the CGTase from *B. circulans* (Matsuura *et al.*, 1984; Buisson *et al.*, 1987; Boel *et al.*, 1990; Klein and

Schulz, 1991).

In region II there are two positions in which only two different amino acid residues are allowed. The third amino acid is either an arginine (R) or a tryptophan (W). The fifth residue is always a carboxylate: aspartic acid (D) or glutamic acid (E). Affinity labelling using glycosyl-epoxides as substrate analogues has led to the identification of the fifth residue as the putative catalytic base in rabbit sucrase and isomaltase (Asp-1396 and Asp-505, respectively) and in human lysosomal α-glucosidase (Asp-518) (Quaroni *et al.*, 1976; Hermans *et al.*, 1991). Mutation of this Asp of lysosomal α-glucosidase to its amide form and mutation of the corresponding Asp residues in the α-amylase from *B. subtilis* and CGTase from *B. sp. 1011* resulted in the loss of enzyme activity. The starch binding capacity of the α-amylase and the CGTase was maintained, suggesting that the Asp is essential for the reaction mechanism (Hermans *et al.*, 1991; Nakamura *et al.*, 1992; Takase *et al.*, 1992). The function of Arg-233 in α-amylase from *B. stearothermophilus* was studied by mutating this residue to Trp and Lys. The conservative change to Lys resulted in a lowering of the specific activity to about 12% of the wild type enzyme (Vihinen *et al.*, 1990). Substitution by Trp, which is the alternative for the third position in region II, resulted in total loss of activity (Holm *et al.*, 1990). In lysosomal α-glucosidase the corresponding Trp-516 was substituted inversely by Arg and also this manipulation resulted in the loss of catalytic function. The residues are evidently not interchangeable despite the fact that only these and no other residues occur alternatively. It is notable that all enzymes which contain only region II conserved, invariable have a Trp on the third position and either an Asp or a Glu on the fifth position, whereas all enzymes which contain all four conserved regions have invariable an Arg on the third position and an Asp on the fifth.

Although glucoamylases contain region II, the homology with other α-glucan degrading enzymes is lower (Svensson, 1988). Affinity labelling studies and site directed mutagenesis have identified Asp-176 as the catalytic base and Glu-179 as the catalytic acid of *A. niger* glucoamylase (Svensson *et al.*, 1990; Sierks *et al.*, 1990).

The last residue of region II is a histidine (H) in almost all of the α-amylases and CGTases. These enzymes need a Ca^{2+} ion for stability and the His has been demonstrated to be involved in the binding of this ion. In *A.niger* α-amylase this function is taken over by the Glu residue (Boel *et al.*, 1990). Several amino acids of the second region have

Table 3: α-glucanases compared in this study.

	ENZYME	SOURCE	ABBRE-VIATION	EC. number	REFER-ENCE
1	α-amylase	Bacillus licheniformis	AMY B.li.	3.2.1.1	1
2	α-amylase	Bacillus stearothermophilus	AMY B.st.	3.2.1.1	2
3	α-amylase	Bacillus amyloliquefaciens	AMY B.am.	3.2.1.1	3
4	α-amylase	Bacillus subtilis	AMY B.su.	3.2.1.1	4
5	α-amylase	Bacillus megaterium	AMY B.me.	3.2.1.1	5
6	α-amylase	Bacillus sp. B1018	AMY B.sp.	3.2.1.1	6
7	α-amylase	Bacillus polymyxa	AMY B.po.	3.2.1.1	7
8	α-amylase	Streptomyces hygroscopicus	AMY S.hy.	3.2.1.1	8
9	α-amylase	Schwanniomyces occidentalis	AMY S.oc.	3.2.1.1	9
10	α-amylase	Xanthomonas campestris pv. campestris	AMY X.ca	3.2.1.1	10
11	α-amylase	Aeromonas hydrophila	AMY A.hy.	3.2.1.1	11
12	α-amylase (MAL S)	Escherichia coli	AMY E.co.	3.2.1.1	12
13	α-amylase (MAL Z)	Escherichia coli	AMY E.co'.	3.2.1.1	13
14	α-amylase (Taka-amylase)	Aspergillus oryzae	AMY TAA	3.2.1.1	14
15	α-amylase (acid)	Aspergillus niger	AMY A.ni.	3.2.1.1	15
16	α-amylase	Dictyoglomus thermophilum	AMY B D.th.	3.2.1.1	16
17	α-amylase	Dictyoglomus thermophilum	AMY C D.th.	3.2.1.1	16
18	α-amylase	Barley	AMY Bar.	3.2.1.1	17
19	α-amylase	Drosophila melanogaster	AMY D.me.	3.2.1.1	18
20	α-amylase	Rat	AMY Rat	3.2.1.1	19
21	α-amylase	Mouse	AMY Mou.	3.2.1.1	20
22	α-amylase	Pig	AMY Pig	3.2.1.1	21
23	α-amylase	Human	AMY Hum.	3.2.1.1	22
24	glucoamylase	Clostridium sp. G0005	GA C.sp.	3.2.1.3	23
25	glucoamylase	Aspergillus niger	GA A.ni.	3.2.1.3	24
26	glucoamylase	Aspergillus oryzae	GA A.or.	3.2.1.3	25
27	glucoamylase	Aspergillus awamori	GA A.aw.	3.2.1.3	26
28	glucoamylase	Rhizopus oryzae	GA R.or.	3.2.1.3	27
29	glucoamylase	Saccharomyces cerevisiae	GA S.ce.	3.2.1.3	28
30	glucoamylase	Saccharomyces diastaticus	GA S.di.	3.2.1.3	29
31	glucoamylase	Saccharomycopsis fibuligera	GA S.fi.	3.2.1.3	30
32	glucoamylase	Schwanniomyces occidentalis	GA S.oc.	3.2.1.3	31
33	lysosomal α-glucosidase	Human	GAA Hum.	3.2.1.3	32
34	oligo-1,6-glucosidase	Bacillus cereus	OG B.ce.	3.2.1.10	33
35	oligo-1,6-glucosidase	Bacillus thermoglucosidasius	OG B.th.	3.2.1.10	34
36	isomaltase	Rabbit	ISO Rab.	3.2.1.10	35

	ENZYME	SOURCE	ABBRE-VIATION	EC. number	REFER-ENCE
37	isomaltase	Human	ISO Hum.	3.2.1.10	36
38	dextranase	Streptococcus mutans	DEX S.mu.	3.2.1.11	37
39	maltase	Saccharomyces carlsbergensis	MAL S.ca.	3.2.1.20	38
40	α-glucosidase	Candida tsukubaensis	AG C.ts.	3.2.1.20	39
41	pullulanase	Klebsiella aerogenes W70	PUL K.ae.	3.2.1.41	40
42	neopullulanase	Bacillus stearothermophilus	NPL B.st.	not classified	41
43	α-amylase-pullulanase	Clostridium thermohydrosul-furicum	APU C.th.	not classified	42
44	sucrase	Rabbit	SUC Rab.	3.2.1.48	35
45	sucrase	Human	SUC Hum.	3.2.1.48	36
46	cylcomaltodextrin hydrolase	Clostridium thermohydrosul-furicum	CD C.th.	3.2.1.54	43
47	maltotetraohydrolase	Pseudomonas stutzeri	G4 P.st.	3.2.1.60	44
48	maltotetraohydrolase	Pseudomonas saccharophila	G4 P.sa.	3.2.1.60	45
49	isoamylase	Pseudomonas amyloderamosa	IAM P.am.	3.2.1.68	46
50	maltohexaohydrolase	Bacillus sp. #707	G6 B.sp.	3.2.1.98	47
51	branching enzyme	Escherichia coli	BRA E.co.	2.4.1.18	48
52	branching enzyme	Synechococcus sp.	BRA S.sp.	2.4.1.18	49
53	branching enzyme	Zea mays L.	BRA Z.ma.	2.4.1.18	50
54	CGT ase	Klebsiella pneumoniae	CGT K.pn.	2.4.1.19	51
55	CGT ase	Bacillus macerans	CGT B.ma.	2.4.1.19	52
56	CGT ase	Bacillus sp. #1011	CGT B.sp.	2.4.1.19	53
57	CGT ase	Bacillus stearothermophilus	CGT B.st.	2.4.1.19	54
58	CGT ase	Bacillus circulans	CGT B.ci.	2.4.1.19	55
59	amylomaltase	Streptococcus pneumonia	AMM S.pn.	2.4.1.25	56

[1]Yuuki *et al.*, 1985; [2]Nakajima *et al.*, 1985; [3]Takkinen *et al.*, 1983; [4]Yang *et al.*, 1983; [5]Metz *et al.*, 1988; [6]Itkor *et al.*, 1990; [7]Uozumi *et al.*, 1989; [8]Hoshiko *et al.*, 1987; [9]Wu *et al.*, 1991; [10] Hu *et al.*, 1992; [11]Gobius & Pemberton, 1988; [12]Schneider *et al.*, 1992; [13]Tapio *et al.*, 1991; [14]Toda *et al.*, 1982; [15]Boel *et al.*, 1990; [16]Horinouchi *et al.*, 1988; [17]Rogers and Milliman, 1983; [18]Boer & Hickey, 1986; [19]MacDonald *et al.*, 1980; [20]Hagenbüchle *et al.*, 1980; [21]Kluh, 1981; [22]Nakamura *et al.*, 1984; [23]Ohnishi *et al.*, 1992; [24]Boel *et al.*, 1984; [25]Hata *et al.*, 1991; [26]Nunberg *et al.*, 1984; [27]Ashikari *et al.*, 1986; [28]Yamashita *et al.*, 1987; [29]Yamashita *et al.*, 1985; [30]Itoh *et al.*, 1987; [31]Dohmen *et al.*, 1990; [32]Hoefsloot *et al.*, 1988; [33]Watanabe *et al.*, 1990; [34]Watanabe *et al.*, 1991; [35]Hunziker *et al.*, 1986; [36]Chantret *et al.*, 1992; [37]Russel & Ferretti, 1990; [38]Hong & Marmur, 1986; [39]Kinsella *et al.*, 1991; [40]Katsuragi *et al.*, 1987; [41]Kuriki & Imanaka, 1989; [42]Melasniemi *et al.*, 1990; [43] Podkovyrov & Zeikus, 1992; [44]Fujita *et al.*, 1989; [45]Zhou *et al.*, 1989; [46]Amemura *et al.*, 1988; [47]Tsukamoto *et al.*, 1988; [48]Baecker *et al.*, 1986; [49]Kiel *et al.*, 1990; [50]Baba *et al.*, 1991; [51]Binder *et al.*, 1986; [52]Takano *et al.*, 1986; [53]Kimura *et al.*, 1987; [54]Sakai *et al.*, 1987; [55]Nitschke *et al.*, 1990; [56]Lacks *et al.*, 1982.

Table 4: Alignment of homologous regions in α-glucanases.

	ENZYME	POS[1]	REGION I	POS[1]	REGION II	POS[1]	REGION III	POS[1]	REGION IV
1	AMY B.li.	100	DVVINH	227	GFRLDAVKH	261	EYWQ	323	FVDNHD
2	AMY B.st.	101	DVVFDH	230	GFRLDAVKH	264	EYWS	326	FVDNHD
3	AMY B.am.	98	DVVLNH	227	GFRIDAAKH	261	EYWQ	323	FVENHD
4	AMY B.su.	97	DAVINH	172	GFRFDAAKH	208	EILQ	264	WVESHD
5	AMY B.me.	109	DLVVNH	202	GFRLDAAKH	247	EVWD	308	FLTNHD
6	AMY B.sp.	135	DFAPNH	225	GIRMDAVKH	257	EWFL	323	FIDNHD
7	AMY B.po.	820	DVVVNH	902	GLRLDTVKH	930	EIFH	990	FIDNHD
8	AMY S.hy.	117	DAVVNH	199	GFRIDAAKH	229	EVIY	286	FVDNWD
9	AMY S.oc.	153	DVVYNH	238	GLRIDSAKH	266	EVYQ	328	FIENHD
10	AMY X.ca.	116	DVVFNH	225	GFRVDAAKH	256	EVIT	323	FAVTHD
11	AMY A.hy.	102	DVVLNH	211	GFRVDAVKH	242	EVIT	309	FAITHD
12	AMY E.co.	310	DVVMNH	456	GFRVDTAKH	503	EAWG	560	YLSSHD
13	AMY E.co'.	246	DGVFNH	332	GWRLDVVHM	373	EHFG	443	QLESHD
14	AMY TAA	117	DVVANH	202	GLRIDTVKH	230	EVLD	292	FVENHD
15	AMY A.ni.[2]	117	DVVPNH	202	GLRIDSVLE	230	EVDN	292	FIENHD
16	AMY B D.th.	231	DFVPNH	304	GYRMDHATG	337	EIVE	398	FLENHD
17	AMY C D.th.	122	DLVVNH	208	GFRLDAAKH	247	EVWD	308	FLRNHD
18	AMY Bar.	101	DIVINH	127	DGRLDWGPH	218	EVWD	299	FVDNHD
19	AMY D.me.	94	DVVFNH	182	GFRVDAAKH	223	EVID	283	FVDNHD
20	AMY Rat	96	DAVINH	190	GFRLDAAKH	230	EVID	292	FVDNHD
21	AMY Mou.	96	DAVINH	193	GFRLDASKH	233	EVID	295	FVDNHD
22	AMY Pig	96	DAVINH	193	GFRIDASKH	233	EVID	295	FVDNHD
23	AMY Hum.	99	DAVINH	196	GFRLDASKH	236	EVID	298	FVDNHD
24	GA C.sp.			453	E-RWEEIGGY				
25	GA A.ni.			176	D-LWEEVNGS				
26	GA A.aw.			201	D-LWEEVNGS				
27	GA A.or.			202	D-LWEEVQGT				
28	GA R.or.			311	D-LWEEVNGV				
29	GA S.ce.			261	D-LWEEVNGM				
30	GA S.di.			529	D-LWEEVNGM				
31	GA S.fi.			234	D-LWEENQGR				
32	GA S.oc.			466	GIWADMNEV				

33	GAA Hum.			514	GMWIDMNEP				
34	OG B.ce.	98	DLVVNH	195	GFRMDVINF	255	EMPG	324	YWNNHD
35	OG B.th	98	DLVVNH	195	GFRMDVINM	256	ETPG	325	YLNNHD
36	ISO Rab.			501	GLWIDMNEV				
37	ISO Hum.			501	GLWIDMNEV				
38	DEX S.mu.	98	DLVVNH	190	GFRMDVIDM	236	ETWG	308	FWNNHD
39	MAL S.ca.	106	DLVINH	210	GFRIDTAGL	276	EVAH	244	YIENHD
40	AG C.ts.			522	GIWLDMNEP				
41	PUL K.ae.	600	DVVYNH	671	GFRFDLMGY	704	EGWD	827	YVSKHD
42	NPL B.st.	242	DAVFNH	324	GWRLDVANE	357	EIWH	419	LLGSHD
43	APU C.th.	488[3]	DGVFNH	594[3]	GWRLDVANE	627[3]	ENWN	699[3]	LLGSHD
44	SUC Rab.			1390	GLWIDMNEP				
45	SUC Hum.			1390	GLWIDMNEP				
46	CD C.th.	238	DAVFNH	320	GWRLDVANE	354	EVWH	416	LIGSHD
47	G4 P.st.	133	DVVPNH	210	GFRFDFVRG	240	ELWK	309	FVDNHD
48	G4 P.sa.	112	DVVPNH	189	GFRFDFVRG	219	ELWK	289	FVDNHD
49	IAM P.am.	291	DVVYNH	370	GFRFDLASV	454	EWSV	502	FIDVHD
50	G6 B.sp.	103	DVVMNH	233	GFRIDAVKH	268	EFWK	329	FVDNHD
51	BRA E.co.	334	DWVPGH	400	ALRVDAVAS	425	EFGG	520	LPLSHD
52	BRA S.sp.	369	DWVPGH	435	GIRVDAVAS	460	EYGG	555	LALSHD
53	BRA Z.ma.	277[3]	DVVHSH	347[3]	GFRFDGVTS	373[3]	EYFS	470[3]	YAESHD
54	CGT K.pn.	130	DYADNH	219	AIRIDAIKH	257	EWFG	328	FMDNHD
55	CGT B.ma	135	DFAPNH	225	GIRFDAVKH	258	EWFL	324	FIDNHD
56	CGT B.sp.	135	DFAPNH	225	GIRVDAVKH	257	EWFL	323	FIDNHD
57	CGT B.st.	131	DFAPNH	221	GIRMDAVKH	253	EWFL	319	FIDNHD
58	CGT B.ci.	135	DFAPNH	225	GIRVDAVKH	257	EWFL	323	FIDNHD
59	AMM S.pn.[2]	188	DKLVYH	286	IVRIDHFRG	316	EWVK	389	YTGTHD

[1]first amino acid position. [2]aligned by hand. [3]counted from the amino-terminus of the mature enzyme.

A.niger and Lys-200 in pig pancreatic α-amylase (Matsuura *et al.*, 1984; Buisson *et al.*, 1987; Boel *et al.*, 1990; Klein and Schulz, 1991).

Also several residues from region III take part in substrate binding: Val-231 in α-amylase from *A.niger* and Ile-235 and Asp-236 in pig pancreatic α-amylase (Matsuura *et al.*, 1984; Buisson *et al.*, 1987). The first residue of region III, glutamic acid (E), is conserved in all enzymes containing region III. Bases on crystallographic data this Glu was proposed to be important for the catalytic function (Matsuura *et al.*, 1984; Klein and Schulz, 1991). The corresponding Glu of α-amylase from *B. subtilis* and of CGTase from *B. sp. #1011* was mutated to Gln to investigate its function. This substitution resulted in exactly the same reduction of activity and retainment of starch binding capacity as was observed by the substitution of the catalytic Asp to Asn in region II, confirming the essential role of Glu in region III (Takase *et al.*, 1992; Nakamura *et al.*, 1992).

A similar effect was obtained when the sixth conserved residue in region IV, aspartic acid (D) was substituted by Asparagine. This Asp was further identified as a catalytic residue based on the crystal structures of the α-amylases from TAA and PIG, and CGTase from *B.circulans*. Also the fifth residue, histidine (H) is extremely well conserved, only α-amylase from *S. hygroscopis* has a Tryptophan (W) at this position of region IV. This His is probably involved in the binding of substrate (Matsuura *et al.*, 1984; Buisson *et al.*, 1987; Klein and Schulz, 1991).

Several enzymes capable of digesting raw starch have in addition to the four regions mentioned above, a common C-terminal sequence motif. This motif was removed from *A. niger* glucoamylase and *B.circulans* α-amylase. The activity of the truncated polypeptides was studied on soluble and raw starch. The enzymes missing the C-terminal domain retained full activity on soluble starch, but were no longer capable to bind to and to digest raw starch. On the other hand, the isolated C-terminal peptide from *B. circulans* could still be absorbed onto raw starch (Svensson *et al.*, 1986; Kim *et al.*, 1992). This C-terminal motif, which consists of 96 to 103 amino acids, has been recognized in several other CGTases (Binder *et al.*, 1986; Kimura *et al.*, 1987; Sakai *et al.*, 1987), in α-amylases (Nakajima *et al.*, 1985; Itkor *et al.*, 1990), in maltotetraose-forming amylase from *P. stutzeri* (Fujita *et al.*, 1989), in glucoamylase from *A.niger* (Boel *et al.*, 1984) and in β-amylase from *Clostridium thermosulfurgenes* (Kitamoto *et al.*, 1988). This motif is the first example of a clear homology between β-amylase and other starch-degrading

enzymes. Most α-amylases, however, do not have this domain, but can still attack raw-starch. Thus, this domain is not universally required for the degradation of raw-starch (Svensson, 1988; Svensson *et al.*, 1989; Itkor *et al.*, 1990).

In summary

Most of the α-glucan degrading enzymes form a family of distantly related proteins and are predicted to have a $(\beta/\alpha)_8$-barrel catalytic domain. They also contain four short regions of homology. These regions play an important role in the binding of the Ca^{2+} ion and the substrate, and several residues take part in the catalytic reaction. Although much is known about the mode of action, there is a disagreement about the assignment of the catalytic residues of the α-amylases. The TAA crystal model suggests that Glu-230 (region III) and Asp-297 (region IV) are the catalytic residues, whereas the PIG crystal model selects Asp-197 (region II) and Asp-300 (region IV) for this role (Matsuura *et al.*, 1984; Buisson *et al.*, 1987).

The glucoamylases, human lysosomal α-glucosidase, rabbit and human sucrase and isomaltase and α-glucosidase from *C. tsukubaensis* contain a short active-site region that compares to region II of the other α-glucanases, but lack the other three homologous regions. They are not predicted by sequence analysis to have the α-amylase type $(\beta/\alpha)_8$-barrel catalytic domain.

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CHAPTER 2

LYSOSOMAL α-GLUCOSIDASE AND GLYCOGEN STORAGE DISEASE TYPE II

LYSOSOMAL α-GLUCOSIDASE
AND
GLYCOGEN STORAGE DISEASE TYPE II

Introduction

Lysosomes and lysosomal storage disorders

Lysosomes are acidic, hydrolase-rich organelles in which biological macromolecules are degraded (deDuve, 1955; 1963). Lysosomes are actively involved in the turnover of cellular components, in the process of cell renewal. Infectious agents such as bacteria are destroyed in the lysosomes of macrophages and polymorphonuclear granulocytes after phagocytosis. Also hormones and a variety of molecules that enter the cell by receptor-mediated endocytosis are degraded in the lysosomes. In spite of the digestive capacity of the lysosomes, the acid hydrolases and their associated (activator) proteins as well as the lysosomal membrane proteins are relatively long-lived.

The concept of lysosomal storage disorders was introduced by Hers in 1965 after his finding of a lysosomal α-glucosidase deficiency in the fatal condition known as Pompe's disease (Hers, 1963; 1965). In case of a genetically determined lysosomal enzyme deficiency, undegraded macromolecular substrates would gradually accumulate within the lysosomes and cause a progressive increase in the size and number of these organelles. The cellular pathology would eventually lead to malfunction of the affected organ. At present the primary defect of more than 30 different lysosomal storage disorders is known (for review see Neufeld, 1991). In by far the majority of disorders it concerns the deficiency of a lysosomal hydrolase. Sometimes the disorder is caused by the deficiency of a lysosomal enzyme activator, or finds its origin in the dysfunction of enzymes, involved in the realization of lysosomal proteins. Salla disease and cystinosis form a separate class of lysosomal storage diseases in that low molecular components accumulate in the lysosomal interior because of a defect in an integral membrane protein instrumental in their export. Lysosomal storage disorders are inherited as autosomal recessive traits, with the exception of Fabry disease and Hunter disease which are X-linked (Scriver *et al.*, 1989).

The estimated incidence of most lysosomal disorders is 1 in 25,000 to 1 in 100,000, but for some diseases, for instance N-acetyl-α-galactosaminidase only 3 patients are known worldwide so far (Van Diggelen *et al.*, 1987; Desnick and Wang, 1990). In certain ethnic groups the frequency of lysosomal disorders is much higher. Examples are Tay Sachs disease among Ashkenazi Jews and French-Canadians, Gaucher disease type I among Ashkenazi Jews and Gaucher disease type III among the Swedish Norrbottnian population. Aspartylglucosaminuria and Salla disease occur frequently among Finns (Aula *et al.*, 1979; Beaudet and Thomas, 1989; Barranger and Ginns, 1989; Sandhoff *et al.*, 1989).

Synthesis and processing of lysosomal enzymes

Lysosomal proteins have a characteristic N-terminal sequence, the signal peptide, that mediates their transport into the lumen of the rough endoplasmic reticulum (RER) membrane. As soon as the signal peptide emerges from the ribosome it is identified by the so called signal recognition particle (SRP) (Walter and Blobel, 1981a; 1981b). Binding of the SRP to the signal peptide results in arrest of translation. The SRP-ribosome complex is then targeted to the RER membrane and attached to it via the SRP receptor, also called the docking protein. The signal peptide interacts with the translocation site in the RER membrane, whereafter the SRP is released and the translation activity restored (Rapoport, 1990). When the protein enters the RER glycosylation starts with the *en block* transfer of an oligosaccharide precursor from dolichol pyrophosphate to predetermined asparagine residues in the sequence Asn-Xxx-Ser/Thr (Xxx may be all amino acids except Pro) (Marshall, 1974; Kornfeld and Kornfeld, 1985). The composition of the lipid linked oligosaccharide precursor is (Dol-P-P)-$(GlcNAc)_2(Man)_9(Glc)_3$.

Once attached to the nascent protein the oligosaccharide structure is subjected to a variety of modifications. High-mannose, hybrid and complex type of N-glycans arise from the differential processing of the $Glc_3Man_9GlcNAc_2$ structure. The modifications start in the RER, and the nature and extent of the processing events in the Golgi apparatus determine the final type of N-linked glycan that is formed.

The terminal glucose residue is removed in the RER by a specific α-1,2

glucosidase (α-glucosidase I). The two remaining glucose residues are subsequently removed by a single α-1,3 specific glucosidase (α-glucosidase II). Both glucosidases are located in the membranes of the RER together with a specific α-mannosidase that catalyses the removal of at least one of the α-1,2-linked mannose residues before the protein is transferred to the Golgi apparatus (Kornfeld and Kornfeld, 1985). Lodish and Kong have suggested that glucose trimming is necessary for the exit of some glycoproteins from the RER (Lodish and Kong, 1984). The newly synthesized lysosomal proteins then are transported to the cis Golgi cisternae and traverse the stack of the Golgi cisternae from the cis through the medial to the trans side by vesicular transport. Most lysosomal enzymes and their activator proteins undergo a highly specific carbohydrate modification, namely the phosphorylation of selective mannose residues. This is accomplished by a phosphotransferase which transfers N-acetylglucosamine 1-phosphate from UDP-N-acetylglucosamine to particular mannose residues (Reitman and Kornfeld, 1981; Waheed *et al.*, 1981). Subsequently, N-acetylglucosamine-1-phosphodiesterase cleaves off the N-acetylglucosamine residue to generate the active phosphomonoester which is called the mannose 6-phosphate recognition marker (Waheed *et al.*, 1981). The phosphodiesterase is probably localized in the mid-Golgi cisternae (Lazzarino and Gabel, 1988). Phosphorylation of the oligosaccharide structure blocks further modification. Unphosphorylated structures are attacked by Golgi mannosidases which stepwise remove the outer five mannose residues. New complex type of structures are then build by the consecutive action of N-acetylglucosaminyl-, fucosyl-, galactosyl-and sialyltransferases of the mid and trans Golgi cisternae. The lysosomal proteins continue their way from the trans Golgi cisternae to the Trans Golgi Network (TGN).

Lysosomal proteins with a mannose 6-phosphate recognition marker bind to mannose 6-phosphate receptors (MPRs). This occurs most likely in the TGN (Griffiths and Simons, 1986; Kornfeld and Mellman, 1989). Two distinct MPRs have been isolated and characterized, the cation-independent and the cation-dependent MPRs. Both are integral membrane glycoproteins with molecular masses of 270 and 46 kD, respectively and they both participate in lysosomal enzyme sorting, although the cation-independent MPR is the dominant receptor in this process (Kornfeld and Mellman, 1989).

The lysosomal enzyme-receptor complex exits from the TGN in a clathrin-coated vesicle (Von Figura and Hasilik, 1986). These vesicles then fuse with an acidic

endosomal compartment (late endosomes), where the low pH induces the lysosomal proteins to be discharged from the MPR. The receptor recycles back to the TGN while the lysosomal proteins enter the lysosomes through fusion of late endosomes and lysosomes.

Although the mannose 6-phosphate receptor pathway is important for lysosomal enzyme targeting, there are alternative mechanisms for transport. Acid phosphatase for instance, does not acquire the mannose 6-phosphate recognition marker, but is transported to the lysosomes via the plasmamembrane, as a transmembrane protein (Waheed *et al.*, 1988; Braun *et al.*, 1989). Glucocerebrosidase is not phosphorylated either and is membrane associated but seems again to follow a different route (Aerts *et al.*, 1986)

Structural and functional features of lysosomal α-glucosidase

Lysosomal α-glucosidase is an exo-acting hydrolase with a pH optimum between 4 and 5. In the lysosomes it is able to degrade glycogen completely because of its capability to cleave both the α-1,4 and the α-1,6 linkages (Jeffrey *et al.*, 1970a; 1970b). Also other α-glucans, as for instance soluble starch and β-limit dextrin can be hydrolysed (see also *Chapter I*). Rabbit muscle lysosomal α-glucosidase was shown to hydrolyse in addition α-1,2 and α-1,3 linkages in disaccharides. The rate of hydrolysis for nigerose (with an α-1,3 linkage) was as high as for maltose (Matsui *et al.*, 1984). The lysosomal α-glucosidase activity is often assayed with the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside. The enzyme is classified as an exo-1,4-α-D-glucosidase (EC 3.2.1.3) since glucose is liberated in the β-configuration.

The human lysosomal α-glucosidase gene (GAA) has been mapped to chromosome 17q21.2 - q23 (Solomon *et al.*, 1979; Nickel *et al.*, 1982; Martiniuk *et al.*, 1985). The gene is approximately 20 kb long and contains 20 exons (Hoefsloot *et al.*, 1990a; Martiniuk *et al.*, 1991a). It is transcribed and spliced in a mRNA of 3.6 kb and codes for a protein of 952 amino acids (Hoefsloot *et al.*, 1988; Martiniuk *et al.*, 1990a).

In vitro translation of mRNA isolated from human liver cells reveals a protein of approximately 100 kD. When canine microsomal membranes are added to mimic entrance in the RER and to allow glycosylation, the apparent molecular mass increases to approximately 110 kD (Van der Horst *et al.*, 1987). An α-glucosidase precursor of the

same size is found in cultured human fibroblasts. The 110 kD precursor contains high mannose type of sugar chains and is phosphorylated and transported to the lysosomes (Hasilik and Neufeld, 1980a; 1980b; Reuser *et al.*, 1985, 1987). A part of the precursor pool is secreted and contains complex next to high mannose type of carbohydrate chains (Wisselaar *et al.*, 1993). The 110 kD precursor is proteolytically processed to a relatively long lived 95 kD species. This occurs in the endosomal compartment or in the lysosomes where also the mature lysosomal α-glucosidase species of 76 and 70 kD are formed (Oude Elferink *et al.*, 1984a; Wisselaar *et al.*, 1993).

Lysosomal α-glucosidase is not only transported to the lysosomes, but is also found at other locations. Application of immunocytochemistry has revealed the presence of lysosomal α-glucosidase at the apical surface of epithelial cells and at the plasmamembrane of transiently transfected COS cells, in which the enzyme was overexpressed (Fransen *et al.*, 1988, Oude Elferink *et al.*, 1989; Hoefsloot *et al.*, 1990b; Willemsen *et al.*, 1991; Klumperman *et al.*, 1991). Membrane association, via an uncleaved signal peptide, has been postulated to cause this unusual localization (Wisselaar *et al.*, 1993). Furthermore, the enzyme is secreted, in precursor form, by most cell types and can be retrieved from the urine. Interestingly, the secreted precursor forms are recognized by the MPR exposed at the plasmamembrane and transported to the lysosomes (Oude Elferink *et al.*, 1984b; Van der Ploeg *et al.*, 1988a, 1988b; Hoefsloot *et al.*, 1990b).

Lysosomal α-glucosidase and glycogen metabolism

Deficiency of lysosomal α-glucosidase results in the accumulation of glycogen in the lysosomes of nearly all types of cells. Therefore the disease associated with this defect has been classified as one of the glycogen storage diseases. The term "glycogen storage disease" covers a group of congenital hereditary diseases characterized by an abnormal aggregation of glycogen in tissues (Hers *et al.*,1989). In 1954 Cori initiated a numerical classification of the glycogen storage diseases (Cori, 1954). At present eight different types of diseases with established enzyme defects are distinguished (Table 1). Figure 1 illustrates at what point the respective enzyme defects interfere with the glycogen metabolism.

Glycogen is metabolized by almost every cell type, however, the control of glycogen metabolism differs per cell type and is adapted to the particular requirements of the cell. Liver and muscle are the two tissues in which glycogen metabolism is the most intense. The liver store of glycogen can provide glucose to the blood for the benefit of other tissues, particularly the brain during short periods of fasting. The blood glucose level is maintained within strict limits by a complex regulatory mechanism of glycogen synthesis and degradation in the liver. Normally the glycogen store in this organ is relatively small and is exhausted after less than a day of fasting. The muscle usually maintains a low glycogen reserve which is mobilized when oxygen or glucose declines during strenuous muscular activity.

The enzymes involved in glycogen storage disease type I, III, V, VI VII and VIII belong to the phosphorolytic pathway of glycogen degradation, and some are tissue specific. The enzyme deficient in GSD IV is not involved in the degradation of glycogen but is a transferase that forms the α-1,6 linkages in glycogen. The glycogen accumulating

Table 1: Numerical classification of glycogen storage diseases.

Type	Enzyme deficiency	Organ involvement	Disease
Ia	glucose 6-phosphatase	liver	Von Gierke's disease
Ib	G-6-P translocase	liver	
II	lysosomal α-glucosidase	muscle	Pompe's disease / generalized glycogenosis
III	debranching enzyme (amylo-1,6-glucosidase)	liver / muscle	Forbe's disease / limited dextrinosis
IV	branching enzyme	liver	Andersen disease / amylopectinosis
V	muscle phosphorylase	muscle	McArdle's disease
VI	liver phosphorylase	liver	
VII	phosphofructokinase	muscle	Tarui disease
VIII	phosphorylase b kinase α	liver	
	phosphorylase b kinase β	liver / muscle	
	phosphorylase b kinase γ		
	phosphorylase b kinase δ		

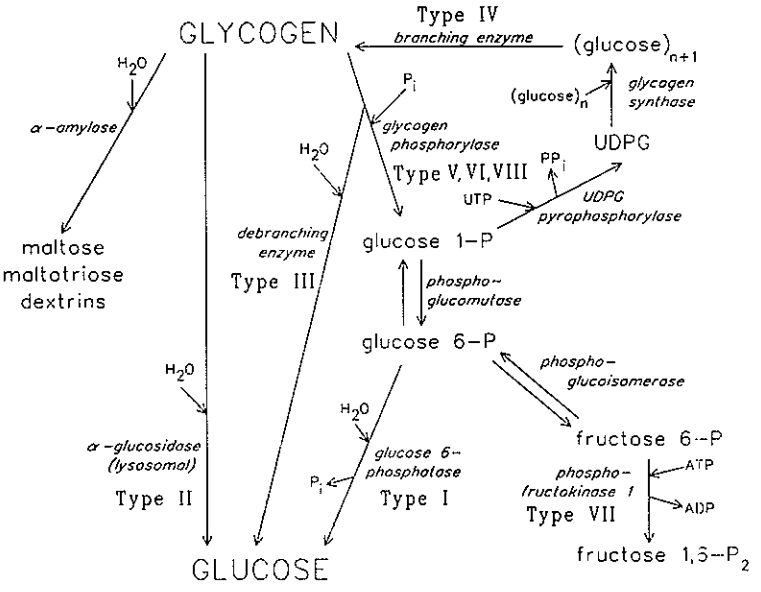

Figure 1 : Schematic representation of glycogen metabolism.
Enzymes involved are printed in Italics. The Roman figures refer to the glycogen storage diseases associated with the enzyme defects. VIII: Glycogen phosphorylase is regulated by phosphorylase b kinase. Deficiency of this kinase leads to GSD VIII.

in this disease is structurally similar to amylopectin. In GSDII the cytoplasmic glycogen metabolism is unaffected but the glycogen that has entered the lysosomes by autophagy starts to accumulate as a result of a lysosomal α-glucosidase deficiency.

Clinical manifestations of glycogen storage disease type II

GSDII (acid maltase deficiency, glycogenosis type II) is clinically heterogeneous. This has led to a somewhat arbitrary distinction of three clinical phenotypes based on age of onset, rate of progression, severity of symptoms, and organ involvement. These are the infantile, juvenile and adult subtypes.

Patients with infantile GSDII (Pompe's disease, generalized glycogenosis) present within the first few months of life with rapidly progressive weakness of the muscles, and enlargement of the heart, liver and tongue. The patients have feeding problems and death

occurs mostly before the age of 2 because of cardiorespiratory failure. Autopsy shows massive intralysosomal accumulation of glycogen (8 to 15% of the wet weight of the tissue) in liver, heart and skeletal muscle.

Juvenile GSDII presents clinically in infancy or early childhood as a myopathy. Respiratory muscles tend to be selectively affected. The disease progresses relatively slowly, and the usual cause of death is respiratory failure. Few patients survive beyond the second decade. Glycogen excess in muscle is less marked and more variable than in infantile GSDII, but in severely affected muscle it can be as high as 10% of the tissue wet weight.

Adult GSDII presents as a slowly progressive myopathy which is frequently associated with respiratory failure. Symptoms usually occur after the age of 20, and some patients have been diagnosed with symptoms starting at the age of 50 (Loonen *et al.*, 1981; Engel *et al.*, 1985; Chancellor *et al.*, 1991). The myopathy affects primarily the torso and limb muscles and may selectively involve individual muscles or isolated areas of a muscle. Death usually results from respiratory failure. Lysosomal glycogen storage is observed in clinically affected muscles but seldomly exceeds 5% of the wet weight of the tissue.

The residual lysosomal α-glucosidase activity determines in general the clinical severity of the disease. Residual enzyme activity is virtually undetectable in infantile GSDII whereas adult patients can have as much as 25% of the normal activity (Mehler and DiMauro,1977; Shanske *et al.*, 1986; Reuser *et al.*, 1978, 1982, 1987; Van der Ploeg *et al.*, 1987, 1988, 1989; Hoefsloot *et al.*, 1990c). Nevertheless, several exceptions have been reported of adult patients with unusually low residual lysosomal α-glucosidase activity (Beratis *et al.*, 1983; Reuser *et al.*, 1987).

Abnormalities in lysosomal α-glucosidase realization

A number of different defects can be envisaged to lead to a deficiency of a lysosomal enzyme. The corresponding gene can be completely or partially deleted. Mutations in the regulatory sequences of the gene can block transcription, whereas splice site mutations can result in defective mRNA. Small insertions and deletions can result in a frame-shift whereby premature stopcodons are introduced. Aberrant mRNA's may be

unstable. Point mutations causing amino acid substitutions can lead to several abnormalities. They may result in the synthesis of an unstable precursor protein, a protein which is impaired in transport and proteolytic processing or in an apparently normal mature protein which is catalytically inactive.

The great diversity of abnormalities in the realization of lysosomal α-glucosidase in cultured fibroblasts and muscle cells of GSDII patients predicts the existence of a multiplicity of mutant alleles. Patients have been described without detectable synthesis of lysosomal α-glucosidase (Beratis *et al.*, 1983; Reuser *et al.*, 1985; Van der Ploeg *et al.*, 1989). Other patients demonstrate a normal synthesis of the lysosomal α-glucosidase precursor but the proteolytic processing to mature enzyme is severely impaired. In one of these latter cases, the precursor seemed to be about 5 kD smaller than the normal precursor of 110 kD (Van der Ploeg *et al.*, 1989). A 108 kD precursor was produced by another patient, due to the lack of a glycosylation site, and there was a phosphorylation defect, in addition (Reuser *et al.*, 1985; Hermans *et al.*, 1993b, *Chapter 5*). Most adult patients synthesize an apparently normal 110 kD precursor, in reduced amount, which then results in a lower than normal amount of mature enzyme in the lysosomes (Reuser and Kroos, 1982; Reuser *et al.*, 1985, 1987). In several of these patients the specific activity and the stability of lysosomal α-glucosidase were not altered (Reuser and Kroos, 1982). These examples illustrate the scala of the abnormalities in the α-glucosidase realization and predict existence of allelic heterogeneity.

The experimental work

The gene coding for lysosomal α-glucosidase and many other lysosomal enzymes have been cloned. Their sequences have revealed some structural features common to all these enzymes (For review see Neufeld, 1991) for instance the N-terminal signal peptide for import of the enzyme into the lumen of the RER and the potential sites for N-linked glycosylation. But the primary sequence is not telling which potential N-linked glycosylation sites are actually used and which of the carbohydrate chains are phosphorylated in the Golgi complex. Also the exact sites of proteolytic cleavage and the location of the catalytic centre and substrate binding sites can not be deduced solely from

the primary sequence.

The experiments described in this thesis were performed to provide more insight in the structure-function relationship of lysosomal α-glucosidase. The studies were aimed at defining the catalytic site (*Chapter 3*), and the glycosylation and phosphorylation sites (*Chapter 4*). A second aim was to identify the mutations responsible for the lysosomal α-glucosidase deficiency in patients with various clinical forms of GSDII (*Chapters 5 - 10*). The following paragraphs provide a brief introduction and discussion of the experimental work.

The catalytic domain

An essential tool for the identification of the catalytic site domain of lysosomal α-glucosidase (*Chapter 3*, Hermans *et al.*, 1991a) has been the active site directed inhibitor conduritol B epoxide (CBE) which had been used before to identify the catalytic site domains of sucrase and isomaltase (Quaroni and Semenza, 1976). CBE acts as a substrate analogue and binds covalently to the essential catalytic site residues. The reaction mechanism is similar to the one described for hydrolases in *Chapter 1* (Figure 2). Because of the structural homology between sucrase, isomaltase and lysosomal α-glucosidase it was assumed that CBE would also act as a substrate analogue for lysosomal α-glucosidase, and therefore could be used to identify the catalytic residue. The kinetics of inhibition were indeed those of an active site directed inhibitor, and with the use of CBE, Asp-518 of lysosomal α-glucosidase was identified as the most likely candidate for being the catalytic base. This conclusion was further supported by the loss of catalytic activity upon substitution of Asp-518 by the amino acid residues Glu, Asn and Gly. The substitutions were introduced by site directed mutagenesis of the cDNA, and their effect was studied by expression of the cDNA in transfected COS cells. The fact that even the most subtle mutation from Asp-518 to Glu is not tolerated suggest that the spatial conformation of the β-carboxyl group at position Asp-518 is mechanistically essential. Other substitutions introduced in the active site domain of lysosomal α-glucosidase were Asp-513 to Glu and Trp-516 to Arg. The former alteration was chosen to study specifically the function of Asp-513, conserved in the α-glucanases evolutionary related to lysosomal α-glucosidase (see *Chapter 1*, Table 2). Quite unexpectedly it appeared that the

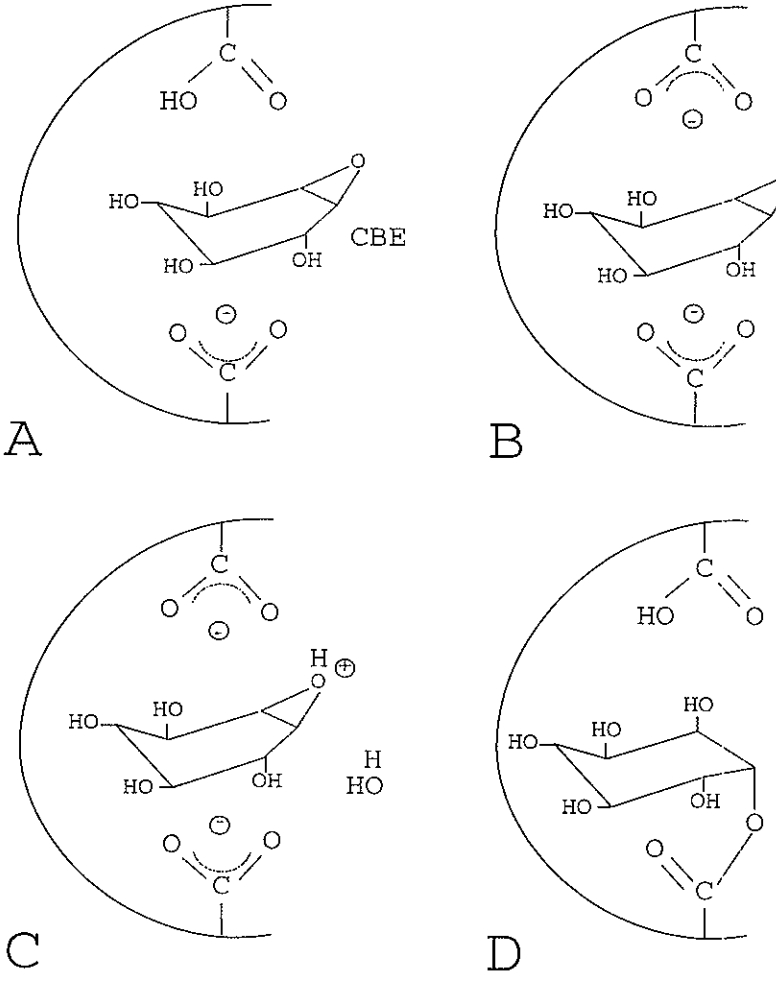

Figure 2 : Schematic representation of the inhibition of lysosomal α-glucosidase by conduritol B epoxide (CBE). CO_2H is the catalytic acid and CO_2^- the catalytic base of lysosomal α-glucosidase.

homologous substitution of Asp-513 by Glu interfered with the post-translational modification of lysosomal α-glucosidase. The Trp-516 to Arg substitution was performed for reason that the α-glucanases containing domains I, II, III and IV have an Arg in domain II at a position where lysosomal α-glucosidase and other α-glucanases with only domain II conserved have a Trp (see *Chapter 1*, Table 4). Loss of activity was observed.

Glycosylation and phosphorylation

Glycosylation on asparagine residues in the sequence Asn-Xxx-Ser/Thr is an important event in the post-translational processing of lysosomal enzymes. Most of these enzymes require a mannose 6-phosphate recognition marker constructed on N-linked oligosaccharide chains for efficient targeting to the lysosomes.

In lysosomal α-glucosidase the actual number of glycosylation sites was determined by individually eliminating the seven potential glycosylation sites through site directed mutagenesis. The influence of each of these glycosylation sites on the transport and catalytic function of the enzyme was investigated and is described in *Chapter 4* (Hermans *et al.*, 1993a). All seven potential sites of lysosomal α-glucosidase are glycosylated. Only elimination of the second glycosylation site at Asn-233 interfered dramatically with the intracellular transport and the maturation of lysosomal α-glucosidase. The exact number of phosphorylation sites could not be established.

In the light of recent findings on lysosomal enzyme glycosylation and phosphorylation the possibility has to be considered that some of the carbohydrate chains of lysosomal α-glucosidase are preferentially phosphorylated, but that phosphorylation of the other sites is not excluded. To give some more information on these fascinating new findings the following three paragraphs give a brief summary of the recent literature.

The glycosylation and phosphorylation of only a few lysosomal proteins had been studied in detail. The results are the following. Four of the five glycosylation sites in β-hexosaminidase β and all three potential sites of β-hexosaminidase α are glycosylated (Sonderfeld-Fresco and Proia, 1989; Weitz and Proia, 1992). No catalytic activity is lost when individual glycosylation sites are deleted. When all three glycosylation sites of β-hexosaminidase α are eliminated the protein is trapped in the lumen of the endoplasmic reticulum and is catalytically inactive. It was found as an insoluble aggregate in a

complex with the resident ER protein BiP (Weitz and Proia, 1992). In both β-hexosaminidase α and β chains the oligosaccharides which are preferentially phosphorylated have been identified. There are two preferred phosphorylation sites in β-hexosaminidase β and one in β-hexosaminidase α. The single site in β-hexosaminidase α corresponds to one of the two sites in β-hexosaminidase β. In cathepsin A (protective protein) both potential sites are glycosylated. Only the carbohydrate chain on the 32 kD subunit is phosphorylated (Morreau *et al.*, 1992). There are three potential glycosylation sites in arylsulfatase A of which only two are normally utilized (Waheed *et al.*, 1983). But when the regular sites are eliminated, the third is used. Glycosylation at sites I and II appeared to be mutually exclusive (Gieselman *et al.*, 1992). Each of the three carbohydrate chains can become phosphorylated when present singly. Gieselman *et al.* hypothesized that the ability of the phosphotransferase to phosphorylate oligosaccharides irrespective of their location in arylsulfatase A mutants indicates that after binding the phosphotransferase searches the surface of the protein for mannose-containing oligosaccharides (Gieselman *et al.*, 1992).

It is now known that the phosphotransferase which initiates the phosphorylation of the mannose 6-phosphate residues in lysosomal enzymes recognizes a conformational determinant on lysosomal enzymes. In cathepsin D this determinant is made up of at least two noncontinuous regions (Lang *et al.*, 1984; Baranski *et al.*, 1990; Baranski *et al.*, 1991). When both these regions of cathepsin D were replaced by homologous regions of the secretory protein glycopepsinogen the resultant chimeric protein was poorly phosphorylated. However, when either of these regions was substituted individually the chimeric proteins were phosphorylated rather well (Baranski *et al.*, 1992). It appeared that this latter phosphorylation was dependent on the presence of amino acid sequences in the amino lobe of procathepsin D. Baranski *et al.* (1992) have postulated two different models to explain these results. In the "independent recognition site" model cathepsin D contains two independent phosphotransferase recognition sites, one on the amino lobe and one on the carboxyl lobe of the protein. The carboxyl lobe would be the most potent recognition site. The "single extended recognition site" model assumes that carboxyl lobe elements as well as regions of the amino lobe form the recognition site. However, any model of phosphotransferase action must solve the problem of how this enzyme can interact with many different lysosomal enzymes. It was suggested that the binding of the

phosphotransferase may involve multiple contact sites extending over a broad surface to the lysosomal enzyme, with only a portion of these required to generate a functional interaction (Baranski *et al.*, 1990). The single extended recognition site model is compatible with this view.

Cantor and Kornfeld (1992) eliminated the natural glycosylation sites of cathepsin D and created new glycosylation sites widely distributed over the surface of the molecule. They found that the oligosaccharides at each glycosylation site were phosphorylated, but that sites located closer to the essential components of the phosphotransferase recognition domain were phosphorylated better than oligosaccharides located further away. These results fit with either model that lysosomal enzymes contain one extended recognition site, or that there are two independent phosphotransferase recognition sites (Baranski *et al.*, 1992; Cantor and Kornfeld, 1992).

Mutation analysis in GSDII

As mentioned in the introduction to the experimental work genetic heterogeneity was likely to occur in GSDII because of the variety of abnormalities observed in the realization of lysosomal α-glucosidase in fibroblasts and muscle cells of patients. To establish the genetic defect(s) and to study the structure-function relationship of lysosomal α-glucosidase, mutation analysis was performed in different types of patients with GSDII. The patients were selected on the basis of an exceptional abnormality in lysosomal α-glucosidase realization or because of an apparent discrepancy between the clinical phenotype and the level of residual enzyme activity.

An American black patient with adult GSDII (GM1935) studied in *Chapter 5* (Hermans *et al.*, 1993b) was first described by Beratis *et al.* (1983) to have a residual lysosomal α-glucosidase activity in fibroblasts of only 1-3% and to be deficient in lysosomal α-glucosidase protein. This was considered unusual since the residual activity tends to be higher in adult GSDII (Mehler and DiMauro,1977; Reuser *et al.*, 1978, 1982, 1987; Van der Ploeg *et al.*, 1987; Hoefsloot *et al.*, 1990c). Upon further analysis the synthesis of lysosomal α-glucosidase appeared to be normal, but the phosphorylation of the precursor was deficient and processing did not occur (Reuser *et al.*, 1985; 1987). In *Chapter 5* it is shown that the molecular mass of the precursor is lower than normal and

that this is caused by the lack of one of the glycosylation sites. Sequence analysis of the lysosomal α-glucosidase gene of the patient revealed four deviations from the known sequence, leading to amino acid substitutions. One substitution (Arg-854 → Stop) was located in a "silent" allele, not expressed at the mRNA level. The other three substitutions (Asp-645 → Glu, Val-816 → Ile and Thr-927 → Ile were located in the second allele. Since the Thr-927 → Ile substitution eliminates the consensus sequence of the seventh glycosylation site (see *Chapter 4*), it explains the lack of an oligosaccharide chain. The remaining two substitutions Asp-645 → Glu and Val-816 → Ile are both conservative and hence it was unpredictable which one would be the disease causing mutation. To make this distinction the mutations were introduced individually in the wild type lysosomal α-glucosidase cDNA via site directed mutagenesis and expressed in COS cells. The Asp-645 → Glu mutation was found to be responsible for the abnormalities in enzyme realization and loss of function, whereas the Val-816 → Ile substitution remained without effect. An increase in the rate of processing of the lysosomal α-glucosidase precursor having the Thr-927 → Ile substitution was observed but the catalytic function of the enzyme was unaffected. Hence, Val-816 versus Ile-816 and Thr-927 versus Ile-927 are polymorphic forms of lysosomal α-glucosidase. The results are at variance with those published by Martiniuk *et al.* (1991b). *Chapter 6* reports on the occurrence of these polymorphisms among seventeen healthy American blacks (Hermans *et al.*, 1993c). The Ile-927 allele occurred with a frequency of 0.21, and the Ile-816 allele with a frequency of 0.18. Ile-816 was in each case linked to Ile-927 in the same allele.

Three patients with a similar type of defect in enzyme realization are described in *Chapters 7 and 8*. In *Chapter 7* it concerns a comparative study of two Dutch patients, one with a juvenile and the other with an adult form of GSDII. The patients were known to have a normal synthesis and phosphorylation of the lysosomal α-glucosidase precursor in cultured fibroblasts but a virtually complete deficiency of processing. The residual enzyme activity in the adult patient was low (3%), but twice as high as in the juvenile patient (Reuser *et al.*, 1985; 1987). By mutation analysis, the juvenile patient turned out to be a genetic compound. One allele had a mutation resulting in the substitution of Pro-545 by Leu. The second allele had a one base pair deletion at position 525 (ΔT-525) causing a frame-shift and resulting in a premature stop codon at position 660-662. The adult patient appeared unexpectedly to be homozygous for the allele coding for the Pro-

545 → Leu substitution. When expressed in COS cells the Δ-525 allele did not result in any detectable enzyme formation and the Pro-545 → Leu substitution resulted in the realization of lysosomal α-glucosidase activity at 8% of the normal level. Since the adult patient has two copies of the Leu-545 allele compared to the juvenile patient who has only one, the mutation analysis explains why the adult patient has twice as much residual lysosomal α-glucosidase activity as the juvenile patient. It is therefore concluded that gene dosage accounts for the different clinical phenotype of the two patients. In *Chapter 8* it concerns another patient with adult GSDII. This patient was identified by mutation analysis as a genetic compound in spite of the fact that her parents were first cousins (Trend *et al.*, 1985). The mutations found are causing the substitutions of Gly-643 by Arg and Arg-725 by Trp. Also these substitutions were proven not to interfere with the synthesis of the lysosomal α-glucosidase precursors but with its intracellular transport and maturation (Hermans *et al.*, 1993d).

The lysosomal α-glucosidase genotypes of four patients with infantile GSDII are described in *Chapters 9 and 10*. The first study (*Chapter 9*) concerns two siblings of a consanguineous Indian family from South Africa. Previously it was shown that the cultured fibroblasts of these patients were completely devoid of lysosomal α-glucosidase activity, and that the lysosomal α-glucosidase precursor although normally synthesised had a decreased molecular mass (Van der Ploeg *et al.*, 1989). The patients appeared to be homozygous for a mutant allele resulting in the substitution of Glu-521 by Lys, three amino acids downstream from the essential carboxylate at Asp-518 (*Chapters 1 and 3*). The inversion of charge introduced by the Glu-521 to Lys substitution in the catalytic site domain results in an abnormal electrophoretic mobility of the patient in SDS-PAGE and is therefore responsible for the decrease in the apparent molecular mass of the precursor. (*Chapter 9*) (Hermans *et al.*, 1991b). A third patient with infantile GSDII also appeared to have an amino acid substitution in the catalytic site domain, namely Met-519 by Thr. The patient was a genetic compound. The second allele was silent. The detailed results are described in *Chapter 10* which also reports on a fourth patient with infantile GSDII. Also this patient had one silent allele and one transcribed allele. The mutation identified in the transcribed allele causes the substitution of Gly-478 by Arg. Expression studies show that neither the Met-519 → Thr, nor the Gly-478 → Arg substitution affects the synthesis of the mutant lysosomal α-glucosidase precursor. However, the catalytic

function is impaired in both cases and the transport from the RER to the Golgi is probably delayed. As a consequence of the latter the secreted mutant precursor molecules have more complex type of oligosaccharide structures than usual and their apparent molecular mass on SDS-PAGE is higher than normal. In all four cases of infantile GSDII investigated the realization of lysosomal α-glucosidase activity is completely lost in accordance with the severe clinical phenotype of the patients.

In addition to the mutation analyses described in this thesis (*Chapters 5-10*) Zhong *et al.* (1991) have identified the mutant alleles of a patient with infantile GSDII. Also this patient was a genetic compound with one silent allele and one allele carrying a point mutation resulting in the substitution of Met-318 by Thr. This substitution was predicted to give major changes in the secondary structure and to create a new potential glycosylation site at Asn-316. It was not investigated whether this new site was actually used.

In the context of mutation analysis an interesting observation was made by Martiniuk *et al.* (1990b) when analyzing the sequence of the GAA2 allele. This is a variant allele of human lysosomal α-glucosidase that encodes for an enzyme with a normal catalytic activity for 4-methylumbelliferyl-α-D-glucopyranoside but a low affinity for glycogen. It concerns by definition a polymorphism since individuals homozygous for this allele do not suffer from GSDII (Swallow *et al.*, 1975; 1989). The sequence alteration compared to the much more frequent GAA1 allele (called wild type sequence in this thesis) is a substitution of Asp by Asn at amino acid position 91. The finding was completely unexpected since Asp-91 is located in the amino terminal propeptide of the lysosomal α-glucosidase precursor, that is no longer present in the mature enzyme. Nevertheless, it affects the affinity of this mature enzyme for glycogen, suggesting that the propeptide has an early decisive effect on the final conformation of lysosomal α-glucosidase (Wisselaar *et al.*, 1993). Figure 3 summarizes the structural and functional aspects of lysosomal α-glucosidase. The sites of proteolytic processing, the location of the catalytic base and the glycosylation sites are indicated. Also included in this figure are the results of mutation analyses. In addition to the mutations shown in Figure 3, silent alleles were identified, each of which could represent an unique mutation. It is notable that the mutations identified so far in the lysosomal α-glucosidase gene are all located between the amino- and carboxylterminal boundaries of the mature enzyme. Considering the striking

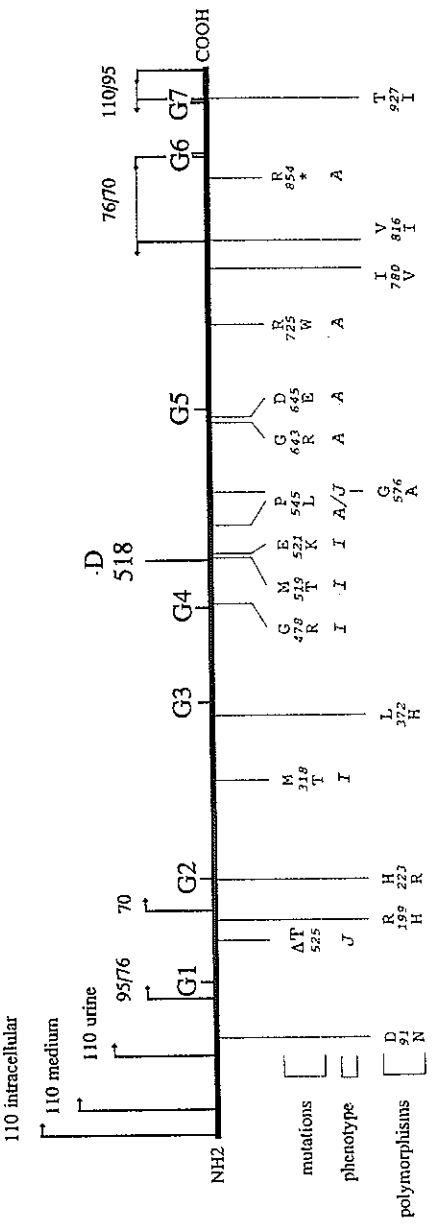

Figure 3: Schematic representation of lysosomal α-glucosidase.
The different processing forms of the enzyme are indicated in kD. The glycosylation sites are symbolized with G. The amino acid residues are shown in the one letter codes. I, J and A represent infantile, juvenile and adult GSDII, respectively.

structural and functional homology between lysosomal α-glucosidase, rabbit and human intestinal sucrase and isomaltase, glucoamylase from *Schwanniomyces occidentalis*, and α-glucosidase from *Candida tsukubaensis*, it is interesting to note that most of the lysosomal α-glucosidase mutations identified so far, are located in highly conserved domains. The allelic constitution of patients with GSDII are summarized in Table 2.

Table 2: Allelic constitution of patients with GSDII.

Phenotype	**Genotype**	**Reference**
Infantile	Lys-521/Lys-521	Hermans *et al.*, 1991b
	Thr-519/silent	*Chapter 10*
	Arg-478/silent	*Chapter 10*
	Thr-318/silent	Zhong *et al.*, 1991
Juvenile	Leu-545/silent (ΔT-525)	*Chapter 7*
Adult	Leu-545/Leu-545	*Chapter 7*
	Glu-645/silent	Hermans *et al.*, 1993b
	Arg-643/Trp-725	Hermans *et al.*, 1993d

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CHAPTER 3

HUMAN LYSOSOMAL α-GLUCOSIDASE: CHARACTERIZATION OF THE CATALYTIC SITE

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Human Lysosomal α-Glucosidase

CHARACTERIZATION OF THE CATALYTIC SITE*

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The substrate analogue conduritol B epoxide (CBE) is demonstrated to be an active site-directed inhibitor of human lysosomal α-glucosidase. A competitive mode of inhibition is obtained with glycogen as natural and 4-methylumbelliferyl-α-D-glucopyranoside as artificial substrate. The inactivation of the enzyme is time and concentration dependent and results in the covalent binding of CBE. Catalytic activity is required for binding to occur. CBE-labeled peptides containing the catalytic residue of lysosomal α-glucosidase were isolated and identified by microsequencing and amino acid analysis. The peptides appeared to originate from a protein domain which is highly conserved among α-amylases, maltase, glucoamylases, and transglucanosylases. Based on the sequence similarity and the mechanism of CBE binding, Asp-518 is predicted to be the essential carboxylate in the active site of lysosomal α-glucosidase. The functional importance of Asp-518 and other residues around the catalytic site was studied by expression of *in vitro* mutagenized α-glucosidase cDNA in transiently transfected COS cells. Substitution of Asp-513 by Glu-513 is shown to interfere with the posttranslational modification and the intracellular transport of the α-glucosidase precursor. The residues Trp-516 and Asp-518 are demonstrated to be critical for catalytic function.

The lysosomal enzyme α-glucosidase (EC 3.2.1.3) hydrolyzes at low pH (pH 4–5) both α-1,4 and α-1,6 linkages in the natural substrates glycogen, maltose and isomaltose (Jeffrey *et al.*, 1970a, 1970b). Deficiency of this enzyme in human and animal species results in glycogenosis type II or Pompe's disease, a recessively inherited lysosomal glycogen storage disorder (Hers, 1963).

Information on the primary structure of lysosomal α-glucosidase has been obtained via molecular cloning and analysis of cDNA and genomic sequences (Hoefsloot *et al.*, 1988, 1990a, 1990b; Martiniuk *et al.* 1990). The cDNA codes for a protein of 952 amino acids with an apparent molecular mass of 110 kDa. The seven potential glycosylation sites of the precursor are all used,[1] and some of the carbohydrate chains are phosphorylated (Hasilik and Neufeld, 1980; Reuser *et al.*, 1985) In addition to glycosylation and phosphorylation, the maturation of α-glucosidase involves proteolytic processing at both the amino- and the carboxyl-terminal ends. It results in the formation of two lysosomal species of 76 and 70 kDa (Reuser *et al.*, 1985; Oude Elferink *et al.*, 1985; Hoefsloot *et al.*, 1988). A molecular species of 95 kDa has been identified as a processing intermediate.

With respect to the functional characteristics of lysosomal α-glucosidase, it was informative to discover a remarkable sequence similarity with the disaccharidases sucrase and isomaltase. Structural conservation of the active site of the three enzymes seemed likely because of overlapping substrate specificities (Hunziker *et al.*, 1986; Hoefsloot *et al.*, 1988). With this in mind we have tested whether conduritol B epoxide (CBE),[2] an active site- directed inhibitor of sucrase and isomaltase (Quaroni *et al.* 1974) could be used to label the catalytic residue of lysosomal α-glucosidase.

CBE is a well known inhibitor of β-glucosidase (Grabowski *et al.*, 1986; Dinur *et al.*, 1986), and its mechanism of action has been described in detail (Legler, 1973; Grabowski *et al.*, 1984). The compound acts as substrate analogue and binds covalently to a carboxylate. Aspartate and glutamate are the preferential catalytic residues (Herrchen and Legler, 1984; Dinur *et al.*, 1986). Sucrase and isomaltase are among the few α-glucosidases shown to be sensitive to inhibition by CBE (Legler, 1973; Quaroni *et al.*, 1974). The inhibitor was shown to bind to isomaltase and sucrase in a ratio of 1 mol of inhibitor/1 mol of enzyme, and more specifically to the β-carboxyl group of the aspartic acid residues at positions 505 and 1249, respectively (Quaroni and Semenza, 1976; Hunziker *et al.*, 1986).

In the present study we have used CBE to identify the catalytic site of lysosomal α-glucosidase, and we have studied the role of potentially important amino acid residues in the catalytic site region by expressing mutagenized α-glucosidase cDNA in mammalian cells.

MATERIALS AND METHODS

Biochemical Assays—Lysosomal α-glucosidase was purified from human placenta as described previously (Reuser *et al.*, 1985). The enzymatic activity was measured with 4-methylumbelliferyl-α-D-glucopyranoside (4-MU) (Reuser *et al.*, 1978) and with glycogen (Koster *et al.*, 1972). Protein concentrations were determined according to Lowry *et al.* (1951).

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[1] M. M. P. Hermans, unpublished results.

[2] The abbreviations used are: CBE, conduritol B epoxide; 4-MU, 4-methylumbelliferyl-α-D-glucopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

The kinetics of enzyme inhibition by CBE was analyzed using 4-MU and glycogen as substrates in 0.2 M sodium acetate, pH 4.3, with 150 and 50 ng of purified placental α-glucosidase, respectively.

To study binding of CBE to human placental α-glucosidase, the inhibitor was added to 10 μg of active or heat-inactivated enzyme in a final concentration of 10 mM, in 20 mM sodium acetate, pH 4.3. Heat inactivation was performed for 1 min at 100 °C. After incubation the different molecular forms of the protein were separated by SDS-PAGE (Laemmli, 1970), and stained with Coomassie Brilliant Blue.

Isolation of CBE-labeled Peptides—Placental α-glucosidase was incubated with CBE (1 μmol/50 μg of protein) in 20 mM sodium acetate, pH 4.3, for 7 h at 37 °C, and then dialyzed against 100 μM ammonium bicarbonate, pH 8.5. The protein was digested with trypsin-L-1-tosyl-amido-2-phenylethyl chloromethyl ketone (Cooper Biomedical) for 24 h at 37 °C. The peptides were recovered by lyophilization and dissolved in 50% acetonitrile and 0.05% trifluoroacetic acid. The samples were subjected to reverse-phase HPLC by using a RP-8 column (Merck). The peptides were eluted with a 0–100% acetonitrile gradient, at a flow rate of 0.5 ml/min. Fractions were collected and the selected peptides were recovered by lyophilization. An aliquot of each sample was used to determine the amino acid sequence of the peptide. The remainder was divided and dissolved in 100 μM ammonium bicarbonate, pH 8.5, for incubation with chymotrypsin (24 h at 37 °C) or in 25 mM ammonium bicarbonate, pH 7.8, for digestion with V8 protease (16 h at 25 °C followed by 4 h at 37 °C). The digests were subjected to reverse-phase HPLC as described above. Isolated peptides were sequenced on an Applied Biosystems Sequencer (477A) on line with a phenylthiohydantoin analyzer (120A). Peptides were hydrolyzed in 6 N HCl, for 24 h at 106 °C, and the amino acid analysis was carried out on a 420A Derivatizer (AB I), on line with a 130A Separation System using the precolumn derivatization method with phenylisothiocyanate.

Construction of Mutants—Site-directed mutagenesis was carried out using the Muta-Gene™ *in vitro* Mutagenesis Kit from Bio-Rad based on the method developed by Kunkel (1985). The oligonucleotides used in this study were synthesized on an Applied Biosystems 381A DNA synthesizer and are listed in Table I.

The full-length human lysosomal α-glucosidase cDNA (Hoefsloot *et al.*, 1988) cloned into M13mp19 was used to transform *Escherichia coli* CJ236 (dut⁻,ung⁻). Single-stranded DNA containing uracil residues was isolated from phages to serve as template in the mutagenesis reaction. The phosphorylated oligonucleotides were annealed to the template. Extension took place with T4 DNA polymerase and ligation with T4 DNA ligase. Small aliquots of the *in vitro* synthesized double-stranded DNA were used to transform *E. coli* DH5αF′ (dut⁺ ung⁺), in which the mutagenized strand is replicated preferentially. Twelve plaques were selected and phages were spotted onto nitrocellulose filters with a slot-blot apparatus. To identify mutants the filter was hybridized at room temperature with the ^{32}P-labeled oligonucleotide used for mutagenesis and was washed at two to three degrees below the estimated melting temperature (Wallace *et al.*, 1981). Mutant cDNAs were cloned in the eukaryotic expression vector pSG5 (Green *et al.*, 1988). The presence of the mutation was verified by double-stranded DNA sequencing with the sequenase™ sequencing kit according to the instructions of the manufacturer (Pharmacia LKB Biotechnology Inc.) using as primers oligonucleotides complementary to the cDNA.

Transient Expression in COS Cells—COS-1 cells (Gluzman, 1981) were cultured in Dulbecco's modification of Eagle's medium supplemented with fetal calf serum (10%) and antibiotics under 10% CO_2, at 37 °C. The transfection protocol was as described before (Hoefsloot *et al.*, 1990a). The cells and the culture medium were harvested at 90 h after transfection. Cell homogenates were made by repeated freezing and thawing of cell pellets in distilled water.

Lysosomal α-glucosidase activity in the cell homogenate and the culture medium was measured with 4-MU and with glycogen substrate as described above.

Immunoblotting—Polyclonal rabbit antibodies against human lysosomal α-glucosidase were used together with *Staphylococcus aureus* membranes (Bethesda Research Laboratories) or protein A-Sepharose beads (Pharmacia) to immunoprecipitate lysosomal α-glucosidase from the cell homogenate and the culture medium. When indicated, immunoprecipitated enzymes were incubated with 10 mM CBE for 7 h at 37 °C in 0.2 M sodium acetate, pH 4.3. The different molecular forms of the protein were separated by SDS-PAGE (Laemmli, 1970) and subsequently blotted onto nitrocellulose filters (Towbin *et al.*, 1979). Lysosomal α-glucosidase was visualized with polyclonal rabbit antibodies against human lysosomal α-glucosidase in combination with ^{125}I-labeled protein A. The blots were exposed to Kodak XAR films.

TABLE I
Oligonucleotides used for mutagenesis

D518	5′ ATGTGGATTGACATGAACGAG 3′ (Asp)
G518	5′ ATGTGGATTGGCATGAACGAG 3′ (Gly)
N518	5′ ATGTGGATTAACATGAACGAG 3′ (Asn)
E518	5′ ATGTGGATTGAAATGAACGAG 3′ (Glu)
R516	5′ GACGGCATGCGGATTGACATG 3′ (Arg)
E513	5′ GCCCTTCGAAGGCATGTGGAT 3′ (Glu)

RESULTS

Binding of CBE to Lysosomal α-Glucosidase—The inhibition of lysosomal α-glucosidase by CBE was studied. Saturable first order kinetics of inactivation were observed, which indicates that a transient reaction intermediate is formed prior to the covalent binding of enzyme and inhibitor (Fig. 1). The Lineweaver-Burk plots show a competitive mode of inhibition. The 1/[S] intercept provides the $-1/K_m$ $(1+[I]/K_i)$ values, in which [I] represents the inhibitor concentration. The calculated K_i value is 11 mM with 4-MU and 7 mg/ml with glycogen as substrate.

The covalent binding of CBE to lysosomal α-glucosidase is further illustrated in Fig. 2. A 7-h incubation of the enzyme with the inhibitor at 37 °C resulted in a mobility shift in SDS-PAGE. A minor part of the enzyme molecules remained unmodified. The mobility shift was not observed when the enzyme was inactivated prior to incubation with CBE (Fig. 2). This illustrates that the observed effect is not solely caused by the presence of CBE in the sample mixture but is actually due to the binding of CBE via a catalytic reaction.

Isolation of CBE-labeled Peptides—To identify the catalytic, CBE-binding residue of lysosomal α-glucosidase, the enzyme was incubated with the inhibitor and subsequently digested

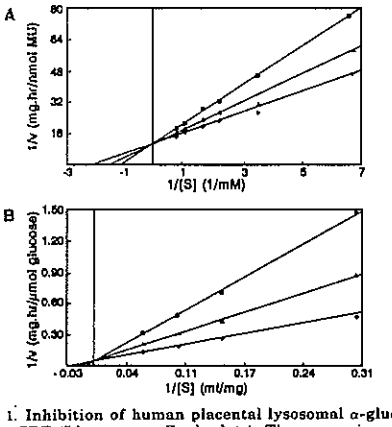

FIG. 1. **Inhibition of human placental lysosomal α-glucosidase by CBE (Lineweaver-Burk plots).** The enzymatic activity was measured with 4-methylumbelliferyl-α-D-glucopyranoside (*A*), and with glycogen (*B*). CBE was added in final concentrations of 0 mM (+), 5 mM (▲) and 10 mM (■).

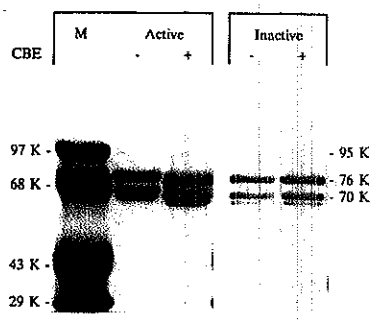

FIG. 2. **The effect of CBE binding on the SDS-PAGE mobility of placental lysosomal α-glucosidase.** Active and heat-inactivated placental α-glucosidase were incubated with 10 mM CBE for 7 h. The different samples were compared by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue. *M*: molecular weight markers.

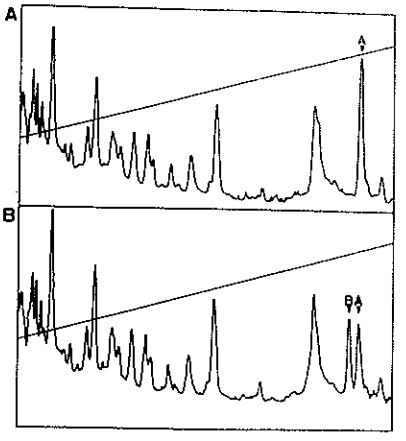

FIG. 3. **HPLC elution profiles of tryptic peptides from lysosomal α-glucosidase.** Human placental α-glucosidase was digested with trypsin before (*A*) or after (*B*) inhibition with CBE. The resulting peptides were separated by HPLC using a RP-8 column. *x* axis, elution time; *y* axis, absorbance (220 nm).

with trypsin. The tryptic peptides were separated by reverse-phase HPLC (Fig. 3*B*). An equal portion of enzyme was subjected to the same procedure without CBE treatment (Fig. 3*A*). The elution profiles appeared to differ in only two aspects. The elution profile of the tryptic peptides derived from CBE-inactivated enzyme (Fig. 3*B*) *versus* active enzyme (Fig. 3*A*) contained an extra peak (marked *B*) and the area of peak A was diminished. The peptides A and B from Fig. 3*B* were isolated, and the sequences of the first seven amino acids were determined by gas-phase microsequencing. Both peptides had the same NH_2-terminal sequence, corresponding to position 480–486 of the previously determined amino acid sequence of lysosomal α-glucosidase (Fig. 4). The appearance of peptide B in addition to A in the tryptic digest is apparently caused by the binding of CBE. Based on the amino acid sequence of lysosomal α-glucosidase and the potential cleavage sites for trypsin, the peptides A and B are expected to end at amino acid residue 527.

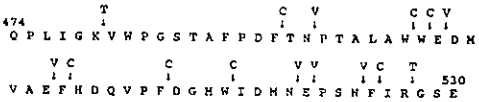

FIG. 4. **Partial amino acid sequence of lysosomal α-glucosidase (Hoefsloot *et al.*, 1988).** The predicted cleavage sites for trypsin (*T*), V-8 protease (*V*), and chymotrypsin (*C*) are indicated.

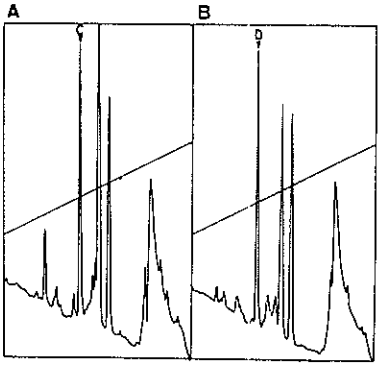

FIG. 5. **HPLC elution profiles of V-8 protease peptides from lysosomal α-glucosidase.** The tryptic peptides indicated in Fig. 3*B* with A and B were isolated and digested with V-8 protease. The resulting peptides were separated as described in Fig. 3. The essential part of the elution profile obtained after digestion of peptide A is shown in the *left panel* (marked *A*). The elution profile obtained after digestion of peptide B is illustrated in the *right panel* (marked *B*). *x* axis, elution time; *y* axis: absorbance (220 nm).

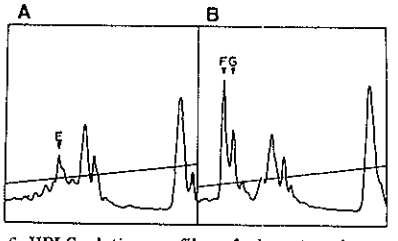

FIG. 6. **HPLC elution profiles of chymotryptic peptides from lysosomal α-glucosidase.** The tryptic peptides indicated in Fig. 3*B* with A and B were isolated and digested with chymotrypsin. The resulting peptides were separated as described in Fig. 3. The essential part of the elution profile obtained after digestion of peptide A is shown in the *left panel* (marked *A*). The elution profile obtained after digestion of peptide B is illustrated in the *right panel* (marked *B*). *x* axis, elution time; *y* axis, absorbance (220 nm).

To zoom in on the region containing the catalytic residue, the tryptic peptides A and B (Fig. 3*B*) were further digested with either V8 protease or with chymotrypsin, and the resulting peptides were analyzed by reverse-phase HPLC. Figs. 5 and 6 show the distinct elution profiles caused by CBE binding. The amino acid sequences of peptides C and D derived by V8 protease digestion of peptide A and B, respectively, were the same (Table II). Thus, the difference in elution time of peptides C and D was attributed to the binding of CBE to peptide D. The amino acid sequence of both peptides starts

TABLE II

Amino acid sequences of chymotrypsin and V8 protease peptides

Peptide C	F H D Q V *X*[a] F D X M
Peptide D	F H X Q V *X* F D G M
Peptide F	D G M *X* I

[a] *X*, unidentifiable amino acid residue.

TABLE III

Amino acid composition of peptide F obtained by chymotrypsin cleavage of tryptic peptide B

Amino acids	No. of residues	
	Estimated from % composition[a]	Deduced from cDNA sequence
Asx	4.4	4
Glx[a]	1.0	1
Ser	0.3	1
Gly	1.4	1
Pro	0.5	1
Met	0.6	2
Ile	0.7	1
Phe	0.8	1
Trp	ND[b]	1
Ala	1.5	0

[a] Glx has been taken as 1.
[b] ND, not determined.

at phenylalanine residue 506 and terminates theoretically at asparagine 520. Also the chymotrypsin fragments E (derived from A) and F and G (derived from B) were isolated. Sequence data were only obtained from peptide F, but more than 4 residues could not be determined due to material wash out at each consecutive sequencing step (Table II). Peptide F starts at position 513 (Fig. 4). To further characterize peptide F, the amino acid composition was determined and compared with the composition as deduced from the cDNA sequence (Table III). The molar concentration of Glx was set at 1. The concentration of some of the amino acid residues was clearly underestimated. But in combination with the sequence data of peptide F this analysis indicated that the chymotrypsin fragment containing the CBE-binding residue starts at Asp-513 and extends to Phe-525. Notably, this implies that chymotrypsin failed to cleave at the potential cleavage site between Trp-516 and Ile-517 (Wilkinson, 1986). The occurrence of alanine could not be explained.

In conclusion, the combined sequence data indicate that the catalytic site of lysosomal α-glucosidase is situated between residue Phe-512 and Glu-521. The sequence of this peptide is strongly conserved among lysosomal α-glucosidase, sucrase, and isomaltase and includes only two carboxylates, *i.e.* at position 513 and 518.

Construction and Expression of Active Site Mutants—*In vitro* mutagenesis was employed to further characterize the role of specific amino acid residues in the catalytic site domain of lysosomal α-glucosidase. To this end the previously cloned 3.6-kilobase α-glucosidase cDNA was inserted in the *Eco*RI site of M13mp19, and specific mutations were introduced with oligonucleotides (see Table I). Based on the results described above and the sequence similarity of lysosomal α-glucosidase, sucrase, isomaltase, and related enzymes (see "Discussion") the following amino acid residues were chosen to be modified. The aspartic acid residue at position 518 was substituted by glutamic acid, leaving the carboxylate intact. More drastic changes were the substitutions of Asp-518 by asparagine or glycine. The second conserved aspartic acid residue in the chymotrypsin fragment containing the catalytic site (at position 513) was altered into glutamic acid. The tryptophan residue at position 516 was replaced by arginine, a residue present at that position in related proteins.

The effect of the mutations was studied by measuring the activity of lysosomal α-glucosidase in transiently transfected COS cells with the artificial 4-MU substrate and with the natural substrate glycogen. As a negative control, COS cells were transfected with a bacterial β-galactosidase construct. The results are presented in Table IV. Transfection with the wild-type cDNA resulted in a significant increase of activity for both substrates. In contrast, no increase of activity was measured after transfection with the mutant cDNA constructs. The mutant enzymes were apparently catalytically deficient. As expected, reversion of Gly-518 to Asp-518 restored the wild-type phenotype. The effect of the different mutations was also evaluated by measuring the α-glucosidase activity secreted by the cells. Also the secreted mutant enzymes appeared to be catalytically deficient (Table IV).

To verify that mutant proteins were actually produced by COS cells, lysosomal α-glucosidase was immunoprecipitated from cell homogenates and culture media and analyzed by Western blotting (Fig. 7). Three forms of lysosomal α-glucosidase were detectable in COS cells transfected with wild-type cDNA: a precursor with a molecular mass of 110 kDa, a processing intermediate of 95 kDa and a mature enzyme of 76 kDa (Fig. 7*A*). A second mature component of 70 kDa present in human placenta was not detectable in the transfected COS cells. The wild-type 110-kDa precursor of α-glucosidase was secreted in the medium (Fig. 7*B*). None of the substitutions of Asp-518 or Trp-516 appeared to affect the synthesis and posttranslational modification of lysosomal α-glucosidase, and the precursor was in each case normally secreted. However, the maturation of the precursor was completely blocked by substitution of Asp-513 by Glu-513 and secretion did not occur. Furthermore, it was observed that each single amino acid substitution leading to an alteration of charge resulted in a change of electrophoretic mobility (Fig. 7, *A* and *B*). This effect was seen when aspartic acid was replaced by asparagine or glycine and when tryptophan was substituted by arginine. Back mutation of Gly-518 to Asp-518 restored the wild-type mobility.

In summary, the results obtained with the active site mutants indicate that the tryptophan residue at position 516 and the aspartic acid residue at position 518 are important for the catalytic function of lysosomal α-glucosidase. The function of the aspartic acid residue at position 513 is more difficult to assess since the substitution by glutamic acid interferes with the maturation process.

Binding of CBE to Active Site Mutants—It was shown in Fig. 2 that catalytic activity is required for binding of CBE.

TABLE IV

Activity of active site mutants of lysosomal α-glucosidase in transiently transfected COS cells measured 90 h after transfection

Type of mutation	Catalytic activity		
	Cells[a]	Medium[a]	Cells[b]
Wild type	313.7	61.2	1070
D518 → N518	18.5	1.1	36
D518 → E518	23.2	0.8	142
D518 → G518	23.0	1.2	85
D518 → G518 → D518	300.4	67.9	2736
W516 → R516	28.3	0.9	133
D513 → E513	70.8	1.4	ND[c]
β-Gal construct	34.6	0.8	290

[a] The activity is expressed as nanomoles 4-MU/milligram protein/hour.
[b] The activity is expressed as nanomoles of glucose/milligram protein/hour.
[c] ND, not determined.

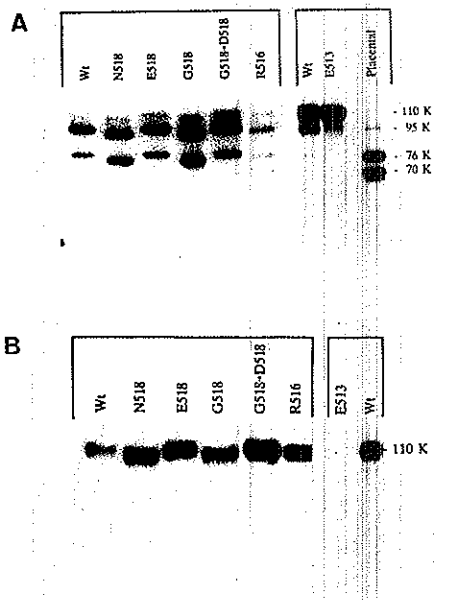

FIG. 7. **Processing of wild-type and mutant lysosomal α-glucosidase in COS cells.** Lysosomal α-glucosidase was immunoprecipitated from transiently transfected COS cells and from the culture medium 90 h after transfection and analyzed by Western blotting after SDS-PAGE. The molecular mass of the wild-type lysosomal α-glucosidase species is indicated. *A*, cells. *B*, media.

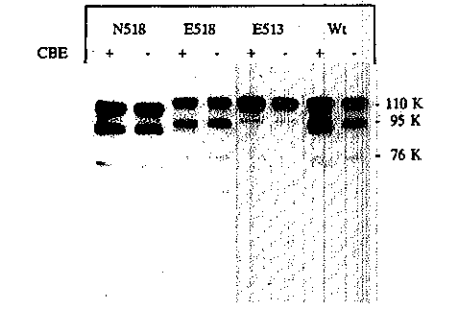

FIG. 8. **The effect of CBE binding on the SDS-PAGE mobility of wild-type and mutant lysosomal α-glucosidase in COS cells.** Lysosomal α-glucosidase was immunoprecipitated from transiently transfected COS-cells and from the culture medium 90 h after transfection and incubated with 10 mM CBE for 7 h. The different samples were compared by SDS-PAGE. The molecular mass of the wild-type lysosomal α-glucosidase species is indicated.

Therefore, all active site mutants should lack CBE binding capacity. This assumption was verified (Fig. 8). As expected, the electrophoretic mobility shift due to CBE binding was only observed for the catalytically active wild-type enzyme. Interestingly, the binding of CBE appeared to be restricted to the 95- and the 76-kDa processed forms of lysosomal α-glucosidase.

DISCUSSION

Two different approaches were taken to identify the catalytic site residues of human lysosomal α-glucosidase. The substrate analogue and active site-directed inhibitor conduritol B epoxide was used to label the essential carboxylate. In this way the catalytic residue was found to be located in a peptide extending from Asp-513 to Asn-520. The second approach involved site-directed mutagenesis to establish the identity of the presumed catalytic residue more definitively.

We demonstrate that CBE inhibits the activity of lysosomal α-glucosidase toward 4-MU-α-D-glucopyranoside and glycogen in a competitive manner. The inactivation is time and concentration dependent and follows Michaelis-Menten kinetics. Using the artificial 4-MU substrate, a K_i of 11 mM was measured. A much lower dissociation constant of 0.17 mM has been reported by Grabowski *et al.* (1986) who studied the effect of CBE on the hydrolysis of 4-MU-β-D-glucopyranoside by human glucocerebrosidase (β-glucosidase). Also other studies point out that CBE has a higher affinity for β- than for α-glucosidases (Legler, 1973; Quaroni and Semenza, 1976; Legler and Harder, 1978; Bause and Legler, 1980).

The reaction mechanism has been studied in detail (Legler, 1973; Braun *et al.*, 1977). It is assumed that a protonated epoxide is formed as a transient intermediate before the epoxide ring of CBE is opened by a carboxylate (Asp or Glu) and a covalent ester bond is established.

The existence of a covalent bond between CBE and lysosomal α-glucosidase is demonstrated by the stability of the bond under strongly denaturing conditions such as used for SDS-PAGE. The mobility shift caused by CBE binding did not occur when heat-inactivated α-glucosidase or catalytically defective mutants were incubated with CBE (Figs. 2 and 8). Thus, catalytic activity is essential for CBE binding. From the fact that the electrophoretic mobility of the wild-type intracellular 110-kDa precursor is not altered by CBE, we conclude that this precursor is catalytically inactive.

The covalent attachment of CBE to the catalytic site residue enabled us to isolate the peptide to which CBE was bound. The HPLC profiles obtained after tryptic digestion of native and CBE-inactivated α-glucosidase appeared to differ by the position of a single peptide. This peptide was identified by microsequencing and was found to start at residue Val-480. The primary sequence deduced from the cloned cDNA predicts that it ends at Arg-527. It includes a region of α-glucosidase which is highly conserved in sucrase and isomaltase, and it contains several aspartic acid and glutamic acid residues which are common binding sites for CBE (Legler, 1973; Quaroni *et al.*, 1974; Herrchen and Legler, 1984; Dinur *et al.*, 1986; Grabowski *et al.*, 1986). By subsequent digestion of this tryptic peptide with V8 protease and with chymotrypsin, the number of potential binding sites could be reduced to two, *i.e.* Asp-513 and Asp-518. The region containing both these residues is almost identical in lysosomal α-glucosidase and in intestinal sucrase and isomaltase (Fig. 9). For rabbit sucrase and isomaltase it was shown that CBE binds to the

```
Lysosomal α-glucosidase : 513 D G H W I D H N E P S N F 525
Human isomaltase        : 500 D G L W I D H N E V S S F 512
Rabbit isomaltase       : 500 D G L W I D H N E V S S F 512
Rabbit sucrase          :1244 D G L W I D H N E P S S F 1256
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FIG. 9. **Amino acid homology around the catalytic site of rabbit sucrase and isomaltase.** The amino acid sequences of lysosomal α-glucosidase (Hoefsloot *et al.*, 1988), human isomaltase (Green *et al.*, 1987), and rabbit sucrase and isomaltase (Hunziker *et al.*, 1986) are aligned. The essential carboxylates of rabbit sucrase and isomaltase are *underlined* (Quaroni and Semenza, 1976).

β-carboxylgroup of the aspartic acid residues Asp-505 (isomaltase) and Asp-1249 (sucrase) in mutually homologous positions. By analogy we expect residue Asp-518 to be the CBE-binding catalytic residue of lysosomal α-glucosidase. Binding of CBE to Asp-518 can possibly explain why chymotrypsin failed to cleave after Trp-516 (one of the favored cleavage sites), whereas it does hydrolyze the peptide bond between Phe-512 and Asp-513. Steric hindrance may be the cause.

To obtain more direct information on the role of potentially important residues in the catalytic site region, amino acid substitutions were made by site-directed mutagenesis at the positions Asp-513, Trp-516, and Asp-518. The nature of all 3 amino acid residues turned out to be critical for catalytic function. One of the most subtle changes, the substitution of Asp-513 by Glu-513, had the most dramatic effect. Both the posttranslational modification and the intracellular transport of α-glucosidase were blocked. The mutant precursor appeared to be synthesized normally but was neither secreted nor converted to mature enzyme. The reason remains obscure as the predicted secondary structure (Chou and Fasman, 1978) and the surface probability (Emini *et al.*, 1985) of the wild-type and the mutant protein do not differ significantly.

The substitutions of Trp-516 by Arg-516 and of Asp-518 by Glu-518, Asn-518, or Gly-518 were more informative, since these mutations did not interfere with enzyme maturation. Considering the mechanism of inactivation (Legler, 1973), the loss of activity caused by the isosteric substitution of Asp-518 by Asn-518 and by the alteration of Asp-518 to Gly-518 can be explained by the loss of the essential carboxyl group. However, it was somewhat unexpected that even the minor change from Asp-518 to Glu-518 deprived α-glucosidase from its catalytic function. This suggests that the spatial conformation of the β-carboxyl group at position Asp-518 is of crucial importance. Elongation of the side chain may prevent substrate binding by steric hindrance. Alternatively, the loss of activity may be caused by the higher pK_a value of glutamic acid compared to aspartic acid which might prevent glutamic acid to act as catalytic base at the enzyme's optimal pH of 4.3. Also, the substitution of Trp-516 (aromatic) by Arg-516 (basic) leads to loss of catalytic function. However, it is virtually excluded that the enzyme inactivation is caused by the covalent binding of CBE to this particular residue. Tryptophan has a non-polar side chain and practically no acidic or basic properties. The inactivation is more likely due to an altered charge distribution. In this context it is notable that Trp-516 is only conserved among the enzymes most related to lysosomal α-glucosidase such as sucrase, isomaltase, and glucoamylases, whereas the α-amylases, maltase, and transglucanosylases have an arginine in their homologous active site regions. In contrast, the carboxylate is conserved throughout (Svensson, 1988).

In conclusion, our results strongly indicate that Asp-518 is the catalytic base of lysosomal α-glucosidase. A second aspartic or glutamic acid residue is expected to act as proton donor. This hypothesis is in line with the recently published data of Onodera *et al.* (1989) showing that rabbit lysosomal α-glucosidase has only one catalytic center with two ionizable groups with pK_a values of 3.6 and 6.1, respectively.

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CHAPTER 4

HUMAN LYSOSOMAL α-GLUCOSIDASE: FUNCTIONAL CHARACTERIZATION OF THE GLYCOSYLATION SITES.

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Human lysosomal α-glucosidase: functional characterization of the glycosylation sites

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N-linked glycosylation is one of the important events in the post-translational modification of human lysosomal α-glucosidase. Phosphorylation of mannose residues ensures efficient transport of the enzyme to the lysosomes via the mannose 6-phosphate receptor. The primary structure of lysosomal α-glucosidase, as deduced from the cDNA sequence, indicates that there are seven potential glycosylation sites. We have eliminated these sites individually by site-directed mutagenesis and thereby demonstrated that all seven sites are glycosylated. The sites at Asn-882 and Asn-925 were found to be located in a C-terminal propeptide which is cleaved off during maturation. Evidence is presented that at least two of the oligosaccharide side chains of human lysosomal α-glucosidase are phosphorylated. Elimination of six of the seven sites does not disturb enzyme synthesis or function. However, removal of the second glycosylation site at Asn-233 interferes dramatically with the formation of mature enzyme. The mutant precursor is synthesized normally and assembles in the endoplasmic reticulum, but immunoelectron microscopy reveals a deficiency of α-glucosidase in the Golgi complex and in the more distal compartments of the lysosomal transport pathway.

INTRODUCTION

Lysosomal α-glucosidase (EC 3.2.1.3) is a glycoprotein like the other acid hydrolases that perform their function in the lysosomes. The function of α-glucosidase is degradation of glycogen to glucose. Enzyme deficiency leads to glycogenosis type II, an inherited glycogen-storage disorder (Hers, 1963). Several patients have been described with a defect in the synthesis or post-translational processing of lysosomal α-glucosidase (Reuser and Kroos, 1982; Beratis et al., 1983; Reuser et al., 1985, 1987; Martiniuk et al., 1986, 1990a; Van der Ploeg et al., 1989; Hermans et al., 1991a; Zhong et al., 1991).

During translation, lysosomal enzymes enter the endoplasmic reticulum where glycosylation is assumed to start even before the protein is completely folded (Rothman et al., 1978). Asparagine residues in the sequence Asn-Xaa-Ser/Thr (Xaa all but Pro) are the potential sites for attachment of N-linked carbohydrate side chains which are transferred *en bloc* from dolichol pyrophosphate (Marshall, 1972; Kornfeld and Kornfeld, 1985). However, not all potential glycosylation sites are used. Studies by Bause and Legler (1981) indicate that a proper protein conformation is required for recognition and glycosylation of a site. After removal of the three terminal glucose residues of the oligosaccharide precursor chain in the endoplasmic reticulum (Kornfeld et al., 1978; Hubbard and Robbins, 1979), most lysosomal enzymes obtain a mannose 6-phosphate recognition marker as lysosomal targeting signal (Creek and Sly, 1984). This is accomplished by the transfer of *N*-acetylglucosamine 1-phosphate from UDP-*N*-acetylglucosamine to particular mannose residues (Reitman and Kornfeld, 1981; Waheed et al., 1981), and the subsequent uncovering of the phosphate by a phosphodiesterase which is probably localized in the mid-Golgi (Lazzarino and Gabel, 1988). Transport to the lysosomes continues via the trans-Golgi cisternae to the trans-Golgi reticulum. Binding to the mannose 6-phosphate receptor in this part of the transport pathway is essential for lysosomal targeting. Lysosomal enzymes are then transported to the late endosomes (Griffiths and Simons, 1986), where a fall in pH causes the ligand to dissociate from the receptor. The enzymes continue their way to the lysosomes and the receptor cycles back to the Golgi complex (Brown et al., 1986; Von Figura and Hasilik, 1986).

Lysosomal α-glucosidase is known to follow this transport route and is subject to several post-translational modifications involving both the carbohydrate chains and the protein backbone. The enzyme is synthesized as a glycosylated precursor of approximately 110 kDa, which is phosphorylated. The amino acid sequence of lysosomal α-glucosidase, as derived from the cloned cDNA, indicates that there are seven potential glycosylation sites (Hoefsloot et al., 1988; Martiniuk et al., 1990b). Proteolytic processing gives rise to a 95 kDa intermediate form and results finally in the formation of two lysosomal enzyme species of 76 kDa and 70 kDa (Hasilik and Neufeld, 1980; Reuser et al., 1985). The latter two forms of lysosomal α-glucosidase have been purified and analysed with respect to their sugar content and carbohydrate chain structure. It was estimated that lysosomal α-glucosidase from human placenta and liver contains an average of four to five carbohydrate chains (Belenky et al., 1979; Mutsaers et al., 1987).

The aim of this study was to establish the actual number of glycosylation sites and to determine the role of each site in the transport and catalytic function of lysosomal α-glucosidase. The approach that was taken was to eliminate the potential sites by site-directed mutagenesis and to study the effect by expression of the mutant cDNA constructs *in vitro* and in transiently transfected COS cells.

MATERIALS AND METHODS

Construction of mutants

The Muta-Gene *in vitro* mutagenesis kit from Bio-Rad (Richmond, CA, U.S.A.) was used to carry out site-directed mutagenesis, as described by Kunkel (1985). The oligonucleotides

Abbreviations used: 4-MU, 4-methylumbelliferyl α-D-glucopyranoside; DMEM, Dulbecco's modification of Eagle's medium; FCS, fetal calf serum.
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Table 1 Oligonucleotides used for mutagenesis

The altered nucleotides are underlined.

Mutant	Oligonucleotide
ΔG1	5'-AAGCTGGAGCAGCTGAGCTCC-3'
ΔG2	5'-GTGCTGCTGCAGACGACGGTG-3'
ΔG3	5'-GTGGTGGAGCAGATGACCAGG-3'
ΔG4	5'-TTCATCACCCAGGAGACCGGC-3'
ΔG5	5'-TTCCTGGGCCAGACCTCAGAG-3'
ΔG6	5'-CTGGCCAGGCAGAACACGATC-3'
ΔG7	5'-CCTGTCTCCCAGTTCACCTAC-3'

used to alter the seven potential glycosylation sites are listed in Table 1. They were synthesized on an Applied Biosystems 381A DNA synthesizer.

Site-directed mutagenesis and cloning of the mutant cDNAs in the eukaryotic expression vector pSG5 (Green et al., 1988) were performed exactly as described previously (Hermans et al., 1991b).

Transient expression in COS cells

COS-1 cells (Gluzman, 1981) were cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics at 37 °C. The transfection protocol was as described previously (Hoefsloot et al., 1990). The culture medium was collected and the cells were harvested 90 h after transfection. Cell homogenates were made by repeated freezing and thawing of cell pellets in distilled water.

The activity of lysosomal α-glucosidase in the cell homogenate and the culture medium was measured with 4-methylumbelliferyl α-D-glucopyranoside (4-MU) as described previously (Reuser et al., 1978). The protein concentrations of cell homogenates were determined with the use of the BCA protein assay kit (Pierce).

Lysosomal α-glucosidase was immunoprecipitated from culture media using a rabbit polyclonal antiserum against human lysosomal α-glucosidase in combination with *Staphylococcus aureus* membranes (Bethesda Research Laboratories) and analysed by immunoblotting (Reuser et al., 1987). To characterize the intracellular forms of lysosomal α-glucosidase, COS cells were labelled for 2 h with [^{3}H]leucine (190 μCi/mmol) (Amersham U.K.) 65 h after transfection, and lysosomal α-glucosidase was immunoprecipitated either directly (pulse) or after 16 h of chase. The different molecular species of lysosomal α-glucosidase were separated by SDS/PAGE (8% acrylamide, 1% cross-link unless indicated otherwise) as described (Reuser et al., 1985).

Phosphorylation of lysosomal α-glucosidase

COS cells were transfected as described above. After 90 h the cells were preincubated for 1 h in phosphate-free DMEM to which FCS, dialysed against 0.9% NaCl, was added in a final concentration of 4%. The medium was then replaced with fresh medium and carrier-free [^{32}P]P_i (Amersham) was added at a concentration of 80 μCi/ml. The cells were pulse-labelled for 6 h and harvested either directly or after a subsequent chase of 12 h. Lysosomal α-glucosidase was immunoprecipitated from cell extracts as described (Reuser et al., 1985) and analysed by SDS/PAGE.

Transcription and translation *in vitro*

Wild-type and mutant cDNAs cloned in the expression vector pSG5 were linearized with *Bgl*II and used as a template in the transcription reaction. The T7 promoter was used for transcription. The reaction was allowed to proceed for 1.5 h at 40 °C in 40 mM Tris/HCl, pH 7.5, containing 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 1 unit of RNAase inhibitor (Promega), 0.8 mg/ml BSA, 500 μM ATP, CTP and UTP, 50 μM GTP, 500 μM dGpppG and 1 μg of DNA template with 20 units of T7 polymerase (Boehringer Mannheim). The template was removed by adding 1 unit of RNAase-free DNAase (Boehringer Mannheim). The excess of nucleotides was removed by Sephadex G-50 filtration.

A 0.2 μg sample of RNA was used for translation *in vitro* in a rabbit reticulocyte lysate system (Promega) containing 54 μCi of [^{35}S]methionine (1130 Ci/mmol; Amersham International) in the presence of canine pancreatic microsomal membranes, according to the instructions of the manufacturer. Radioactively labelled lysosomal α-glucosidase was immunoprecipitated and separated by SDS/PAGE as described above.

Immunocytochemistry

Immunocytochemistry on transiently transfected COS cells was performed exactly as described previously (Hoefsloot et al., 1990).

RESULTS

In lysosomal α-glucosidase, asparagine residues in the recognition sequence for N-linked glycosylation, Asn-Xaa-Thr/Ser (Xaa can be any residue except Pro), are found at seven positions, namely at Asn-140, -233, -390, -470, -652, -882 and -925 (Hoefsloot et al., 1988; Martiniuk et al., 1990b) (EMBL entry number Y00839). These potential glycosylation sites were eliminated one by one to determine which sites were actually used, and to examine the importance of individual sites for enzyme function and lysosomal targeting. To this end the recognition consensus sequence of

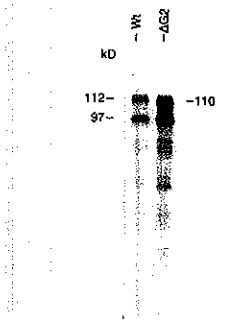

Figure 1 Translation *in vitro* of human lysosomal α-glucosidase

Wild-type (Wt) and mutant (ΔG2) cDNAs were transcribed *in vitro* and the mRNAs were translated in a rabbit reticulocyte lysate in the presence of canine pancreatic microsomes. The proteins were labelled with [^{35}S]methionine, immunoprecipitated and separated by SDS/PAGE. Lane 1, wild type; lane 2, mutant ΔG2. The molecular masses (kDa) of the unglycosylated and glycosylated lysosomal α-glucosidase precursors are indicated.

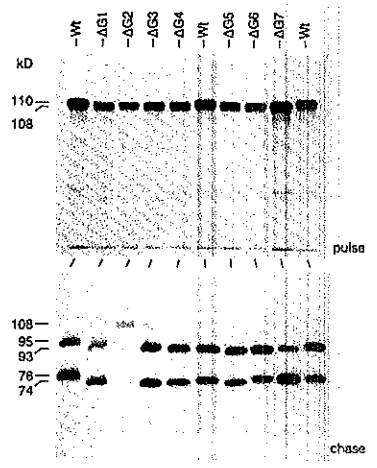

Figure 2 Synthesis and processing of wild-type and mutant lysosomal α-glucosidase in COS cells

COS cells transfected with wild-type (Wt) and mutant cDNA constructs were labelled for 2 h with [^{3}H]leucine and harvested directly (Pulse) or after a subsequent period of 16 h (Chase). Lysosomal α-glucosidase was immunoprecipitated and analysed by SDS/PAGE. The molecular masses (kDa) of wild-type and mutant lysosomal α-glucosidase species are indicated.

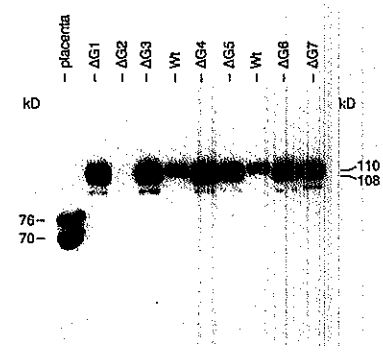

Figure 3 Secretion of wild-type and mutant lysomal α-glucosidase by transiently transfected COS cells

Lysosomal α-glucosidase was immunoprecipitated from the culture media at 90 h after transfection and analysed by Western blotting after SDS/PAGE. The molecular masses (kDa) of wild-type (Wt) and mutant lysosomal α-glucosidase species are indicated.

each potential site was altered by site-directed mutagenesis of the lysosomal α-glucosidase cDNA inserted in M13mp19. The codon for Asn was replaced by one coding for the very similar amino acid residue Gln, using the oligonucleotides listed in Table 1. The mutants, each missing a different glycosylation site, were designated ΔG1–ΔG7 in order of appearance from the N-terminus.

Transcription and translation *in vitro*

The use of glycosylation sites was tested by cloning the wild-type and mutant cDNAs in the eukaryotic expression vector pSG5 allowing *in vitro* transcription and subsequent translation of the mRNAs in the presence of dog pancreatic microsomes. Translation of wild-type mRNA resulted in the formation of two molecular species, a translocated and glycosylated precursor of 112 kDa and an unglycosylated precursor of 97 kDa (Figure 1; and Van der Horst et al., 1987). The glycosylation sites are easily identified by this method. Figure 1, for instance, shows that the substitution of Asn-233 by Gln-233 (ΔG2) results in a 2 kDa size decrease in the glycosylated precursor, whereas the apparent size of the unglycosylated wild-type and mutant precursor remains the same (97 kDa).

Transient expression in COS cells

The functional consequence of the removal of glycosylation sites was investigated by following the synthesis and maturation of α-glucosidase in transiently transfected COS cells. [^{3}H]Leucine-labelled wild-type and mutant lysosomal α-glucosidase species were immunoprecipitated from cell homogenates after a 2 h pulse period or after a subsequent chase of 16 h, and analysed by SDS/PAGE (Figure 2). The secreted form of lysosomal α-glucosidase was immunoprecipitated from the culture media and analysed by Western blotting (Figure 3). In cells transfected with wild-type cDNA the lysosomal α-glucosidase precursor of 110 kDa is synthesized (pulse) and converted into a 95 kDa intermediate and a 76 kDa mature species (chase) (Figure 2). The culture medium contains only the secreted 110 kDa precursor (Figure 3). All mutant precursor proteins (isolated from cells and media) missing one potential glycosylation site appeared to have a slightly lower molecular mass (108 kDa) than the wild-type precursor. This indicates that all seven potential glycosylation sites of lysosomal α-glucosidase are used. For the mutants lacking the first, third, fourth or fifth glycosylation site (ΔG1, ΔG3, ΔG4 and ΔG5), the lower molecular mass is maintained during the maturation process leading to the formation of a 93 kDa processing intermediate and a 74 kDa mature enzyme species (Figure 2, Chase). A different effect was observed with the mutants ΔG6 and ΔG7. The precursor and the processing intermediate were smaller (93 kDa) than the comparable wild-type species, but the final maturation products of ΔG6 and ΔG7 (76 kDa) and wild-type lysosomal α-glucosidase were of the same size (Figure 2, Chase). This indicates that a C-terminal peptide containing the glycosylation sites at Asn-882 and Asn-925 is cleaved off when lysosomal α-glucosidase matures from 95 to 76 kDa.

The only glycosylation site which has a severe effect on the biosynthesis of α-glucosidase when eliminated is Asn-233 (Figure 2). The mutant precursor of 108 kDa is evidently formed during the 2 h pulse, but the 93 kDa processing intermediate and the 74 kDa mature enzyme are clearly deficient in the chase. The ΔG2 precursor is not secreted into the culture medium, in contrast with all other mutant precursors which are secreted normally (Figure 3).

To investigate the effect of glycosylation on the catalytic activity of lysosomal α-glucosidase, cell homogenates and culture media of transfected COS cells were assayed for enzyme activity. COS cells transfected with *Escherichia coli* β-galactosidase cDNA were taken as a reference. The data of a typical transfection

Table 2 Activity of the glycosylation mutants of lysosomal α-glucosidase in transiently transfected COS cells measured 90 h after transfection

The activity is expressed as nmol of 4-MU/h per mg of protein. The activities compared with wild-type are given within parentheses as percentages ± S.E.M. ($n = 1$–4).

Type of mutation	Catalytic activity (nmol/h per mg)			
	Cells		Medium	
Wild-type	960	(100)	406	(100)
ΔG1	665	(61.7 ± 7.4)	175	(42.6 ± 5.5)
ΔG2	116	(5.0 ± 2.3)	15	(2.1 ± 0.9)
ΔG3	789	(77.9 ± 9.1)	307	(89.2 ± 15.7)
ΔG4	671	(69.8)	275	(67.4)
ΔG5	645	(66.8 ± 6.6)	152	(89.1 ± 30.1)
ΔG6	919	(90.1 ± 7.7)	304	(75.9 ± 5.2)
ΔG7	1027	(109.2 ± 6.9)	473	(116.6 ± 0.1)
β-Gal construct	50	(0)	3	(0)

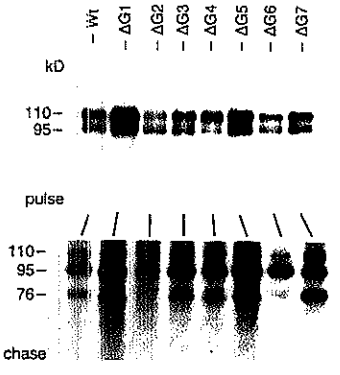

Figure 4 Phosphorylation of wild-type and mutant lysosomal α-glucosidase in COS cells

COS cells transfected with wild-type (Wt) and mutant cDNA constructs were labelled for 6 h with [^{32}P]P_i and harvested directly (pulse) or after a subsequent period of 12 h (chase). Lysosomal α-glucosidase was immunoprecipitated from the cell homogenates and separated by SDS/PAGE (10% acrylamide, 1% cross-link). The molecular masses of the wild-type lysosomal α-glucosidase species are indicated.

experiment and the average outcome of the different experiments are given in Table 2. When compared with wild-type, all glycosylation-site-defective mutants except ΔG2 exhibited intracellularly a similar activity of lysosomal α-glucosidase. ΔG2 was different in that only 5% of wild-type activity was measured in the cell homogenates and less than 3% in the medium. Near-normal levels of lysosomal α-glucosidase were secreted by COS cells transfected with the other mutant constructs (Table 2).

Phosphorylation of lysosomal α-glucosidase

To determine which of the seven oligosaccharide chains of lysosomal α-glucosidase were phosphorylated, we transfected COS cells with the wild-type and mutant cDNA constructs and

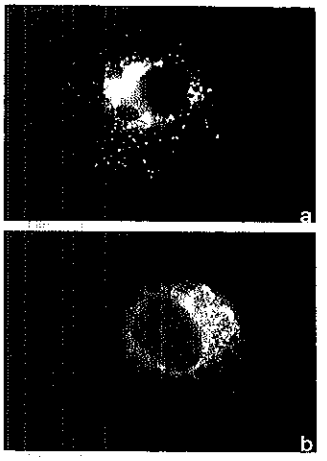

Figure 5 Localization of glycosylation-site-deficient lysosomal α-glucosidases in transfected COS cells studied by light microscopy

Transiently transfected cells were fixed and incubated with a rabbit polyclonal antiserum against human lysosomal α-glucosidase. Immune complexes were visualized with goat anti-rabbit IgG conjugated to fluorescein. (a) ΔG4, (b) ΔG2. Magnifications: (a) × 227, (b) × 567.

labelled the cells with [^{32}P]P_i. After a 6 h labelling period, phosphorylated precursor and intermediate forms of lysosomal α-glucosidase were observed in cells expressing either wild-type or mutant enzyme (Figure 4). The mature forms of lysosomal α-glucosidase became detectable after a subsequent chase of 12 h, except in cells expressing ΔG2. The ^{32}P-labelling procedure appeared sensitive enough to demonstrate conversion of the ΔG2 precursor into the 93 kDa intermediate but the formation of mature (74 kDa) enzyme remained undetectable. When [^{32}P]P_i-labelled wild-type lysosomal α-glucosidase was incubated with endoglycosidase F, before SDS/PAGE, no phosphorylated protein could be detected, indicating that the [^{32}P]P_i was linked to the mannose residues of the carbohydrate side chains (results not shown).

Intracellular transport

The intracellular localization of the mutant α-glucosidase species missing one glycosylation site was investigated using immunocytochemistry. As observed in earlier studies, the formation of mature enzyme was in all instances correlated with a typical punctate lysosomal labelling pattern. Figure 5(a), for instance, illustrates the lysosomal localization of ΔG4. Transfection of COS cells with ΔG2, showing an apparent maturation defect, resulted in a diffuse network of labelled structures spreading from the nucleus into the cytoplasm (Figure 5b). The exact intracellular localization of ΔG2 was revealed by immunoelectron microscopy (Figure 6). Labelling of the nuclear envelope and the endoplasmic reticulum was obtained but the enzyme could not be detected in the Golgi complex nor in the trans-Golgi reticulum or the lysosomes. The latter compartments were labelled after transfection with the wild-type cDNA construct (results not shown).

Figure 6 Intracellular localization of ΔG2 in transfected COS cells studied by immunoelectron microscopy

Ultrathin cryosections were incubated with rabbit polyclonal antiserum against human lysosomal α-glucosidase and subsequently with goat anti-rabbit IgG coupled to colloidal gold. Mutant lysosomal α-glucosidase was found at the nuclear envelope (**a**) and in the endoplasmic reticulum (**b**), but not in the Golgi complex (**c**), or the lysosomes (**d**). N, nucleus; M, mitochondrion; R, rough endoplasmic reticulum; G, Golgi complex; L, lysosomes. Magnifications: (**a**) × 37400, (**b**) × 34160, (**c**) × 51935, (**d**) × 26550.

DISCUSSION

Site-directed mutagenesis was applied to determine the glycosylation sites of lysosomal α-glucosidase and to define their significance for enzyme function and lysosomal targeting. The removal of one high-mannose type of carbohydrate chain is expected to result in a molecular mass decrease of approximately 2 kDa and to lead to a comparable increase in electrophoretic mobility on SDS/PAGE. This effect was observed on deletion of each of the seven potential glycosylation sites of the 110 kDa precursor. The mobility shift in the more mature forms of α-glucosidase is informative with respect to the sites of proteolytic processing. The 2 kDa size decrease is maintained after formation of the 95 kDa processing intermediate irrespective of the deleted glycosylation site. This implies that the peptides that are cleaved off during the conversion of 110 kDa into 95 kDa do not contain a glycosylation site. Since the seventh glycosylation site is located just 31 residues from the C-terminus of the 110 kDa precursor (Hoefsloot et al., 1988), we conclude that the proteolytic conversion occurs mainly at the N-terminus of the precursor. On further maturation of the enzyme to 76 kDa, the mutants lacking the sixth and the seventh glycosylation sites at Asn-882 and Asn-925 are no longer smaller than the wild-type species whereas the other mutants are still decreased in size. Thus the maturation from 95 to 76 kDa involves C-terminal processing at a site upstream of Asn-882. On the basis of the N-terminal sequence of the 76 kDa form of lysosomal α-glucosidase and the sequence of C-terminal tryptic peptides (Hoefsloot et al., 1988), we estimate that the site is located between amino acid residues 820 and 880.

According to the amino acid sequence of lysosomal α-glucosidase, the first glycosylation site is located between the N-termini of the 76 and 70 kDa species purified from human placenta. This leaves four sites occupied in the 70 kDa species. These findings are in line with the earlier estimates of Mutsaers et al. (1987) on the number of carbohydrate chains attached to human placental lysosomal α-glucosidase. A figure of four to five side chains per polypeptide molecule was obtained for a preparation containing equimolar amounts of the 76 and 70 kDa species.

Six of the seven mutants, each lacking a different glycosylation site, are transported to the lysosomes and are catalytically active. Also the proteolytic processing and secretion of these mutants is normal. However, elimination of the second glycosylation site at Asn-233 has a dramatic effect. The precursor (108 kDa) is synthesized normally but the intermediate and mature forms of α-glucosidase are severely decreased in amount. The relative persistence of the ΔG2 precursor during the 16 h chase (Figure 2) suggests a delayed transport of the mutant precursor from the endoplasmic reticulum to the Golgi. This suggestion is sustained by the fact that the immunogold labelling of the endoplasmic reticulum for lysosomal α-glucosidase is quantitatively normal, whereas the Golgi complex and the more distal compartments of the lysosomal transport route are clearly deficient in lysosomal α-glucosidase. This explains why transfection of COS cells with ΔG2 does not lead to a significant increase in the lysosomal α-glucosidase activity.

Mutation analyses of the glycosylation sites of a few other lysosomal proteins have been reported. For example, site-directed mutagenesis was used to determine the essential glycosylation sites of human β-hexosaminidase B (Sonderfeld-Fresco and Proia, 1989). In this enzyme four of the five potential sites were found to be glycosylated. Elimination of each site individually had no effect on lysosomal targetting or catalytic function. In addition to these studies, two naturally occurring glycosylation-site mutations in lysosomal proteins have been reported. One concerns arylsulphatase A. Normally, two of the three potential sites are glycosylated, but individuals carrying the pseudo-deficiency allele have lost one of the two utilized sites by a mutation changing Asn-350 to Ser (Gieselman et al., 1989). However, the introduction of this mutation into wild-type cDNA did not affect the catalytic function or the stability of the encoded enzyme. The decreased arylsulphatase A activity leading to this pseudodeficiency is caused by a mutation abolishing the first polyadenylation signal. As a consequence, the mRNA species is labile which explains the severely diminished rate of enzyme synthesis (Gieselman et al., 1989). The second mutation of a glycosylation site was found in saposin B. The mutation changes Thr-23 to Ile which eliminates the only possible site. The mutant protein is believed to be rapidly degraded (Rafi et al., 1990; Kretz et al., 1990). These examples and our own experiments demonstrate the differential function of glycosylation sites.

The incorporation of [^{32}P]P_i in the different biosynthetic forms of lysosomal α-glucosidase allows us to make an estimate on the minimal number of carbohydrate chains that are phosphorylated. Although each mutant is missing a different glycosylation site, it appears that all mutant precursor proteins are phosphorylated. From this we conclude that at least two of the seven carbohydrate chains contain the mannose 6-phosphate recognition marker.

Furthermore, it is evident that at least one of the five carbohydrate chains located within the boundaries of the 76 kDa polypeptide is phosphorylated, since the mature enzyme is also phosphate-labelled. The maturation defect of mutant ΔG2 prevents the assessment of phosphorylation at site Asn-233 and thereby hampers a more detailed analysis of the phosphorylation sites by site-directed mutagenesis. Further identification of the phosphorylation sites via sequence analysis of phosphorylated peptides is in progress.

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CHAPTER 5

CONSERVATIVE SUBSTITUTION OF ASP-645 → GLU IN LYSOSOMAL α-GLUCOSIDASE AFFECTS TRANSPORT AND PHOSPHORYLATION OF THE ENZYME IN AN ADULT PATIENT WITH GLYCOGEN STORAGE DISEASE TYPE II.

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The conservative substitution Asp-645 → Glu in lysosomal α-glucosidase affects transport and phosphorylation of the enzyme in an adult patient with glycogen-storage disease type II

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Glycogen-storage disease type II (GSDII) is caused by the deficiency of lysosomal α-glucosidase (acid maltase). This paper reports on the analysis of the mutant alleles in an American black patient with an adult form of GSDII (GM1935). The lysosomal α-glucosidase precursor of this patient has abnormal molecular features: (i) the molecular mass is decreased, (ii) the phosphorylation is deficient and (iii) the proteolytic processing is impaired. Sequence analysis revealed four mutations leading to amino acid alterations: Asp-645 → Glu, Val-816 → Ile, Arg-854 → Stop and Thr-927 → Ile. By using allele-specific oligonucleotide hybridization on PCR-amplified cDNA we have demonstrated that the Arg-854 → Stop mutation is located in one allele that is not expressed, and that the other allele contains the remaining three mutations. Each of the mutations was introduced in wild-type cDNA and expressed in COS cells to analyse the effect on biosynthesis, transport and phosphorylation of lysosomal α-glucosidase. The Val-816 → Ile substitution appeared to have no significant effect in contrast with results [Martiniuk, Mehler, Bodkin, Tzall, Hirshhorn, Zhong and Hirschhorn (1991) DNA Cell Biol. 10, 681–687] and was therefore defined as a polymorphism. The Thr-927 → Ile substitution deleting one of the seven glycosylation sites was found to be responsible for the decrease in molecular-mass, but not for the deficient proteolytic processing and phosphorylation. It did not cause the enzyme deficiency either. The third mutation leading to the Asp-645 → Glu substitution was proven to account in full for the observed defects in transport, phosphorylation and proteolytic processing of the newly synthesized α-glucosidase precursor of the patient.

INTRODUCTION

Lysosomal glycogen is normally degraded by lysosomal α-glucosidase (acid maltase; EC 3.2.1.3), and accumulates when this enzyme is deficient (Hers, 1963). The disease, known as glycogenosis type II or glycogen-storage disease type II (GSDII), is inherited as an autosomal recessive trait. The clinical manifestations can vary considerably with respect to time of onset and severity of symptoms (McKusick, 1990).

Apart from clinical heterogeneity, there is also a high degree of biochemical and molecular heterogeneity. A multitude of mutant alleles has been suspected from the molecular abnormalities of the lysosomal α-glucosidase species in cultured fibroblasts and muscle cells from several patients (Reuser et al., 1978, 1985, 1987; Beratis et al., 1978, 1983; Reuser and Kroos, 1982; Van der Ploeg et al., 1989; Hoefsloot et al., 1990a). Genetic heterogeneity has been demonstrated more directly by Southern- and Northern-blot analysis (Martiniuk et al., 1986, 1990a; Hoefsloot et al., 1988; Reuser et al., 1988).

The first two reports on mutation analysis in GSDII have appeared recently (Hermans et al., 1991a; Zhong et al., 1991). In both cases it concerned a patient with a severe infantile form of the disease. We discovered in two sibs from a consanguineous couple a glutamic acid to lysine substitution at position 521 in the homozygous form. The mutation was confirmed to be responsible for the increased electrophoretic mobility of the mutant enzyme precursor, the deficient proteolytic processing and catalytic activity, and the defective intracellular transport (Hermans et al., 1991a). Substitution of threonine for methionine at amino acid position 318 was found by Zhong et al. (1991) in one of the two lysosomal α-glucosidase alleles of their patient. The mutation gives major changes in the predicted secondary structure and creates a potential N-linked glycosylation site, but it remains to be investigated whether the new site is actually used. The second allele of the patient was not expressed.

In this study we report on a case of adult GSDII, which was deliberately chosen for its interesting complexity. Fibroblasts from this American black patient (cell line GM1935) were originally investigated by Beratis et al. (1983). The residual activity of lysosomal α-glucosidase was found to be unusually low compared with the relatively mild clinical phenotype of the patient. When we studied the biosynthesis of lysosomal α-glucosidase in cell line GM1935, two molecular abnormalities were observed (Reuser et al., 1985, 1987). First, the 110 kDa lysosomal α-glucosidase precursor was not processed to mature enzyme. Second, phosphorylation of the precursor was deficient. The latter abnormality prevents formation of the mannose 6-phosphate recognition marker which is assumed to be essential for lysosomal targeting (Creek and Sly, 1984). Indeed, the amount of enzyme in the lysosomes was found to be extremely low.

In an attempt to explain these findings we have now analysed the genetic phenotype of the patient. Different mutations were detected in the two lysosomal α-glucosidase alleles. One mutant allele with a premature stop codon was found to be silent. The second allele appeared to harbour two polymorphisms in addition

Abbreviations used: GSDII, glycogen-storage disease type II; 4-MU, 4-methylumbelliferyl α-D-glucopyranoside; 1 × SSC, 0.15 M NaCl/0.015 M sodium citrate; 1 × SSPE, 15 mM NaCl/10 mM NaH_2PO_4/1 mM EDTA.

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to the mutation causing the abnormal properties of the patient's lysosomal α-glucosidase.

The same patient was studied independently by Martiniuk et al. (1991) who reported on the silent allele and ascribed the lysosomal α-glucosidase deficiency to a Val → Ile substitution at amino acid position 816. Two other substitutions in the same allele (Asp-645 → Glu and Thr-927 → Ile) were identified as polymorphisms. The analysis described in this paper, however, predicts the Asp-645 → Glu substitution to be the mutation and the Val-816 → Ile substitution to be a polymorphism.

MATERIALS AND METHODS

Pulse–chase experiments

The fibroblast cell line GM1935 was obtained from the Human Mutant Cell Repository Institute for Medical Research, Camden, NJ, U.S.A. The control cell line from a healthy individual was from Dr. M. F. Niermeijer, Department of Clinical Genetics, University Hospital, Rotterdam, The Netherlands.

Cells were cultured in Dulbecco's modification of Eagle's medium, supplemented with 10% fetal calf serum and antibiotics, under 10% CO_2, at 37 °C. Labelling conditions with [H]leucine or carrier-free [^{32}P]P_i were as described by Reuser et al. (1985). Cells were pulse-labelled for 2 or 6 h and harvested either directly or after a subsequent chase of 9 h. In some experiments, tunicamycin (final concentration 10 μg/ml) was added 1 h before labelling and during labelling to inhibit glycosylation of newly synthesized proteins. Lysosomal α-glucosidase was immunoprecipitated from cell extracts and analysed by SDS/PAGE as described (Hasilik and Neufeld, 1980; Reuser et al., 1985).

DNA amplification and sequencing

DNA and RNA were extracted from cultured fibroblasts by standard procedures (Sambrook et al., 1989). cDNA synthesis was carried out using Amersham's cDNA Synthesis System Plus and oligo(dT) primers. PCR amplification using intronic or exonic primers specific for lysosomal α-glucosidase was as described (Hermans et al., 1991a). Each 100 μl of PCR reaction mixture contained approx. 0.5 μg of DNA, 100 pmol of each primer and 2 units of *Taq* polymerase (BRL) in the standard PCR buffer recommended by Sambrook et al. (1989). Amplification was performed in a Perkin–Elmer Cetus DNA amplifier. The first cycle was: 5 min denaturation at 94 °C, 5 min annealing at 59 °C and 5 min DNA synthesis at 72 °C. Subsequent cycles (25–30) consisted of 2 min at 94 °C, 2 min at 59 °C and 5 min at 72 °C. The DNA-synthesis step of the final cycle was extended to 15 min. In some instances, PCR-amplified DNA fragments were cloned in a T-tailed Bluescript plasmid vector. This so-called T-vector was constructed as described by Marchuk et al. (1991). DNA fragments were sequenced using the T7 polymerase sequencing kit (Pharmacia LKB Biotechnology Inc.) with appropriate primers.

Slot-blot analysis and allele-specific oligonucleotide hybridization

PCR products were denatured in 0.4 M NaOH/25 mM EDTA for 10 min and applied directly to Hybond N$^+$ membranes using a slot-blot apparatus. The filters were air-dried, prehybridized for 1 h at 42 °C in 5 × SSPE/1% SDS/50 μg/ml salmon sperm DNA, and subsequently hybridized for 3 h in the same solution to ^{32}P-labelled oligonucleotides. The filters were washed in 6 × SSC for 5 min at 2–3 °C below the estimated melting temperature (Wallace et al., 1981).

Site-directed mutagenesis

Site-directed mutagenesis was carried out as described by Hermans et al. (1991b). The following oligonucleotides, synthesized on an Applied Biosystems 381A DNA synthesizer, were used: Glu-645, TCGGGGCCGAAGTCTGCGGCT; Ile-816, ACACCATCAACATCCACCTCC; Stop-854, TGGAGAGGCCTGAGGGGAGCT; Ile-927, TCCAACTTCATCTACAGCCCC.

Transient expression in COS cells

Wild-type and mutant cDNAs were cloned in the eukaryotic expression plasmid pSG5. The transfection of COS cells was carried out as described by Hoefsloot et al. (1990b). The activity of lysosomal α-glucosidase in the cell homogenates and the culture media was measured with 4-methylumbelliferyl α-D-glucopyranoside (4-MU) and with glycogen as described by Hermans et al. (1991b). Radioactive labelling and analysis of lysosomal α-glucosidase expressed in COS cells was as mentioned above.

Immunocytochemistry

Immunocytochemistry on transiently transfected COS cells was performed exactly as described before (Hoefsloot et al., 1990b).

RESULTS AND DISCUSSION

Delineation of the molecular phenotype

The lysosomal α-glucosidase activity in the fibroblast cell line GM1935 derived from a black American adult with GSDII is 1–3% of the average control value (Beratis et al., 1983; Reuser et al., 1985, 1987). Figure 1(a) reveals the molecular nature of the enzyme deficiency. In control fibroblasts, the enzyme is synthesized as a precursor of approximately 110 kDa, and is proteolytically processed to a long-lived 95 kDa intermediate. In a later stage, mature enzyme of 76 and 70 kDa is formed (Hasilik and Neufeld, 1980; Oude Elferink et al., 1985; Reuser et al., 1985). These major molecular forms of lysosomal α-glucosidase

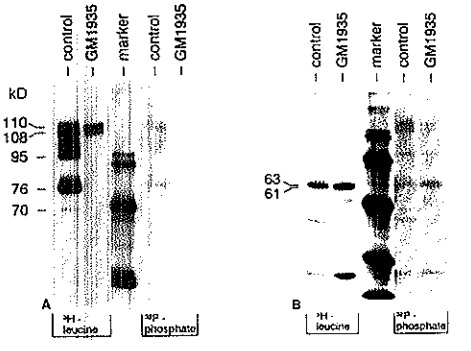

Figure 1 Labelling of lysosomal α-glucosidase (a) and lysosomal β-hexosaminidase (b) with [^{3}H]leucine or [^{32}P]P_i in control and GM1935 fibroblasts

The fibroblasts were labelled for 6 h with [^{3}H]leucine or [^{32}P]P_i and harvested directly. Lysosomal α-glucosidase (a) or β-hexosaminidase (b) were immunoprecipitated from the cell homogenates and analysed by SDS/PAGE. The molecular masses (kDa) of wild-type and mutant protein species are indicated.

are labelled in a 6 h pulse period with [^{3}H]leucine (Figure 1a, lane 1). The Figure reveals, in addition, short-lived processing intermediates with molecular masses between 110 kDa and 95 kDa, which are not always observed. From Figure 1(a) it is evident that the post-translational modification of the lysosomal α-glucosidase precursor is impaired in cell line GM1935. The mutant precursor is, in addition, about 2 kDa smaller than normal (108 kDa). A deficient phosphorylation of the mutant precursor became apparent when the labelling was performed via incorporation of ^{32}P into the mannose 6-phosphate recognition marker. A phosphorylation defect was not observed when the [^{3}H]leucine and [^{32}P]P, incorporation were compared for the reference lysosomal enzyme β-hexosaminidase (Figure 1b). It is therefore an intrinsic feature of the mutant lysosomal α-glucosidase precursor.

These results confirm our earlier findings except that the decrease in molecular mass of the mutant precursor escaped our attention in the high-percentage polyacrylamide gels used previously (Reuser et al., 1985).

A decrease in the apparent molecular mass of a polypeptide can have several causes. It can result from a deletion, an aberrant splicing of the transcript or a point mutation introducing a premature stop codon. Also a mutation causing the loss of a glycosylation site has to be considered. We have demonstrated recently that even a single amino acid substitution can give rise to a significant difference in the electrophoretic mobility of lysosomal α-glucosidase on SDS/PAGE (Hermans et al., 1991a,b). The possible lack of a glycosylation site was assessed by comparing the size of the unglycosylated and glycosylated wild-type and mutant precursors. To this end, glycosylation was inhibited with tunicamycin. Figure 2 illustrates that the wild-type and mutant lysosomal α-glucosidase precursors are the same size (97 kDa) when synthesized in the presence of tunicamycin. On the basis of these results our working hypothesis was that the lysosomal α-glucosidase precursor of mutant GM1935 lacks one of the seven glycosylation sites. In the preceding paper (Hermans et al., 1993), we have demonstrated by site-directed mutagenesis that individual elimination of six of these seven sites does not lead to defective processing and loss of enzyme function. Only the loss of the glycosylation site at amino acid position 233 was found to interfere with enzyme maturation and transport, but the phosphorylation was not affected. Therefore we conclude that the lack of a glycosylation site alone cannot account for the abnormalities observed in cell line GM1935.

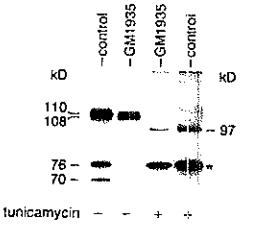

Figure 2 Labelling of lysosomal α-glucosidase with [^{3}H]leucine in the presence or absence of tunicamycin

The fibroblasts were labelled for 6 h with [^{3}H]leucine in the presence (+) or absence (−) of tunicamycin and harvested directly. Lysosomal α-glucosidase was immunoprecipitated from the cell homogenates and analysed by SDS/PAGE. The molecular masses (kDa) of wild-type and mutant lysosomal α-glucosidase species are indicated. Lanes 1 and 4, control; lanes 2 and 3, GM1935. The asterisk indicates a molecular species of unknown identity that is only formed in the presence of tunicamycin.

Sequence analysis

To identify the mutations responsible for the molecular phenotype of GM1935, we PCR-amplified all exonic regions of the lysosomal α-glucosidase gene. Most PCR products were sequenced directly. In some instances, however, it was necessary to clone the PCR fragments before sequencing. In these cases at least four clones were analysed.

Sequence analysis of the complete coding region revealed several differences from the wild-type cDNA sequence (Hoefsloot et al., 1988; Martiniuk et al., 1990b) (EMBL entry number Y00839). All these differences were heterozygous, indicating that GM1935 carries two distinct lysosomal α-glucosidase alleles. Besides several known and two new polymorphisms, we discovered four mutations leading to an amino acid alteration: an Asp to Glu substitution at amino acid position 645 in exon 14, a Val to Ile substitution at position 816 in exon 17, a Thr to Ile substitution at position 927 in exon 19, and a premature stop at position 854 in exon 18 (Table 1). Except for the last mutation, the same substitutions were reported by Martiniuk et al. (1991).

Since several mutations were found, the alleles had to be defined more precisely. We started out with the Val-816 → Ile, the Arg-854 → Stop and the Thr-927 → Ile mutations. Exons

Table 1 Base-pair substitutions: comparison with the lysosomal α-glucosidase cDNA sequence in EMBL/Genbank (Y00839)

Nucleotide position is counted from the initiation codon of the precursor. References: 1, Hoefsloot et al. (1990b); 2, Martiniuk et al. (1990b).

Nucleotide position	Exon	cDNA	GM1935	Amino acid alteration	Reference
Mutations					
1935	14	C	A	Asp-645 → Glu	
2446	17	G	A	Val-816 → Ile	
2560	18	C	T	Arg-854 → Stop	
2780	19	C	T	Thr-927 → Ile	
Polymorphisms					
596	3	G	A	Arg-199 → His	1,2
1203	8	A	G	–	2
1374	9	C	T	–	
1581	11	A	G	–	1,2
2553	18	A	G	–	1,2
2862	20	G	A	Non-coding	

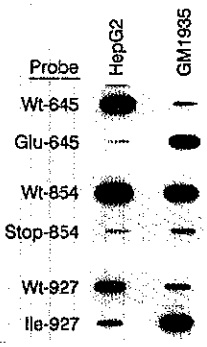

Figure 3 Allele-specific oligonucleotide hybridization of PCR-amplified cDNA

PCR products were denatured and applied to Hybond N⁺ membranes using a slot-blot apparatus and probed with ^{32}P-labelled oligonucleotides for the presence of either normal (Wt) or mutant cDNA sequences

16–19 containing the latter three mutations were PCR-amplified in one fragment from genomic DNA and this fragment was cloned. Six different clones were sequenced. Three were found to have the Arg-854 → Stop mutation in combination with a wild-type sequence at position 816 and 927. The other three clones were wild-type at position 854, but contained both the Val-816 → Ile as well as the Thr-927 → Ile mutation. From these results we conclude that the Val-816 → Ile and the Thr-927 → Ile mutations are located together in one allele, and the Arg-854 → Stop mutation in the other.

The substitution of Ile for Thr-927 eliminates the consensus sequence (Asn-Phe-Thr) for N-linked glycosylation of the seventh glycosylation site. Therefore this mutation is expected to be responsible for the lower molecular mass of the GM1935 lysosomal α-glucosidase precursor. The mutation resulting in the premature stop of enzyme synthesis at amino acid position 854 leads hypothetically to a protein of approximately 98 kDa, because 98 amino acids and two glycosylation sites are lacking. We did not detect a lysosomal α-glucosidase polypeptide of this size in cell line GM1935, but such a mutant protein might be unstable. Another reason for the absence of a 98 kDa polypeptide in GM1935 could be that the allele carrying this mutation is not expressed.

To investigate whether both alleles of GM1935 were expressed, we extracted RNA from the cells and reverse-transcribed it into cDNA. We then PCR-amplified a fragment spanning all four mutations. Allele-specific oligonucleotide hybridization of slot-blots was performed to screen for the presence of the mutations. cDNA from GM1935 was found to hybridize with the Glu-645 and the Ile-927 oligonucleotides, but not with the Stop-854 oligonucleotide. When oligonucleotides complementary to the wild-type sequences at positions 645, 854 and 927 were used, hybridization was only obtained at position 854. cDNA amplified from RNA extracted from human HepG2 cells hybridized only with the wild-type oligonucleotides (Figure 3). As suitable positive and negative controls for hybridization conditions, plasmid constructs containing the wild-type and mutant sequences were used (results not shown). Two conclusions can be drawn from these results. First, the GM1935 allele characterized by the Arg-854 → Stop mutation is not expressed. Second, the allele which we found to harbour the Val-816 → Ile and the Thr-927 → Ile mutations also contains the Asp-645 → Glu mutation, and is expressed at the RNA level. Thus this second allele must encode the mutant protein. This is in agreement with the results reported by Martiniuk et al. (1991) which were obtained with a different methodology.

The question remains of which of the mutations is responsible for the defect in post-translational modification. The Thr-927 → Ile mutation eliminating the glycosylation site at Asn-925 seems unlikely, since we have demonstrated that the loss of this site does not interfere with enzyme processing or function (Hermans et al., 1993). Moreover, the Chou–Fasman and the Garnier algorithms did not predict a significant change in the secondary structure of the protein (Chou and Fasman, 1978; Garnier et al., 1978). No significant differences were observed either when the Val-816 → Ile substitution was analysed. In contrast, the replacement of Asp-645 by Glu disturbs a β-strand according to the Chou–Fasman algorithm.

The mutations and their effects

To study the effects of the various mutations on the biosynthesis and function of lysosomal α-glucosidase, they were introduced in the wild-type cDNA by site-directed mutagenesis and expressed in COS cells. Besides single amino acid substitutions, a double mutant was made containing both the Asp-645 → Glu and the Thr-927 → Ile substitutions. At least two independent clones of each construct were tested.

COS cells were harvested 60 h after transfection and the lysosomal α-glucosidase activity was determined with the artificial 4-MU substrate and with the natural substrate glycogen. At least three transfection experiments were performed for each mutation. Typical results of one such experiment are given in Table 2 which also includes data on the average outcome of three independent experiments. COS cells transfected with *Escherichia coli* β-galactosidase cDNA were used as a negative control. Transfection with the wild-type, the Val-816 → Ile or the Thr-927 → Ile cDNAs resulted in a significant expression of activity for both substrates, indicating that these substitutions represent polymorphisms rather than mutations. In contrast, the activity measured after transfection with the Asp-645 → Glu or the double-mutant construct was hardly above background. The small additive effect of the Thr-927 → Ile on the Asp-645 → Glu substitution was not significant. No increase in activity was measured after transfection with cDNA containing the Arg-854 → Stop mutation.

Our results are at this point at variance with those reported by Martiniuk et al. (1991) who expressed their constructs in SV40-immortalized human fibroblasts. In the one experiment they report on, the Thr-927 → Ile substitution was without effect, the Asp-645 → Glu substitution resulted in a 67% decrease in lysosomal α-glucosidase activity, and the enzyme activity in fibroblasts transfected with the Val-816 → Ile mutant cDNA was not above background.

The synthesis and proteolytic processing of the wild-type and mutant lysosomal α-glucosidase species were assayed by pulse–chase labelling at 60 h after transfection. The results are illustrated in Figure 4. In COS cells transfected with either the wild-type cDNA or the cDNA containing the Val-816 → Ile mutation, the usual 110 kDa precursor was synthesized in a 2 h pulse with [^{3}H]leucine. COS cells transfected with the Thr-927 → Ile mutant cDNA produced a 108 kDa precursor. Transfection with the Asp-645 → Glu mutant construct resulted in the synthesis of an apparently normal 110 kDa precursor in the 2 h

Table 2 Catalytic activities of lysosomal α-glucosidase mutants in transiently transfected COS cells 60 h after transfection

The activity is expressed as nmol of 4-MU/h per mg of protein with MU as substrate or nmol of glucose/h per mg of protein with glycogen as substrate. The activities compared with the wild-type are given within parentheses as percentages ± S.E.M. ($n = 3$).

Type of mutation	Catalytic activity: 4-MU (nmol/h per mg)		Glycogen (nmol/h per mg)	
COS cells	106.8	(0)	360	(0)
Wild-type	582.7	(100)	2180	(100)
Asp-645 → Glu	133.8	(2.9 ± 2.7)	340	(1.5 ± 1.5)
Val-816 → Ile	535.6	(105.0 ± 14.9)	1832	(74.4 ± 6.4)
Arg-854 → Stop	113.0	(1.3 ± 1.2)	320	(0)
Thr-927 → Ile	245.2	(68.0 ± 38.9)	1830	(72.4 ± 8.4)
Asp-645 → Glu/Thr-927 → Ile	122.4	(1.6 ± 1.6)	240	(0.5 ± 0.5)

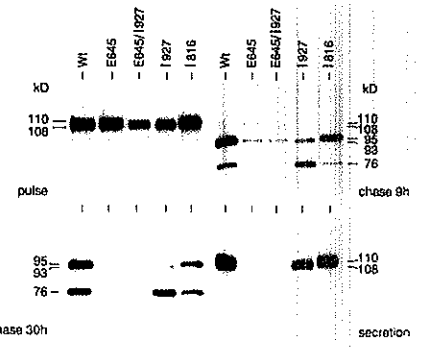

Figure 4 Synthesis and processing of wild-type and mutant lysosomal α-glucosidase species in transfected COS cells

COS cells transfected with wild-type or mutant cDNA constructs were labelled for 2 h with [^{3}H]leucine and harvested directly (pulse) or after a subsequent period of either 9 or 30 h (chase). Lysosomal α-glucosidase was immunoprecipitated from the cell homogenates and analysed by SDS/PAGE. To study secretion, lysosomal α-glucosidase was immunoprecipitated from the chase media. The molecular masses (kDa) of wild-type and mutant lysosomal α-glucosidase species are indicated.

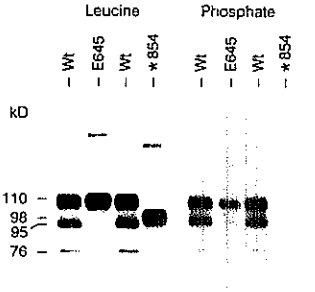

Figure 5 Labelling of wild-type and mutant lysosomal α-glucosidase species in transfected COS cells with [^{3}H]leucine and with [^{32}P]P_i

COS cells transfected with wild-type or mutant cDNA constructs were labelled for 6 h with [^{3}H]leucine or [^{32}P]P_i and harvested directly. Lysosomal α-glucosidase was immunoprecipitated from the cell homogenates and analysed by SDS/PAGE. The molecular masses (kDa) of wild-type and mutant lysosomal α-glucosidase species are indicated.

pulse. The double-mutant precursor was decreased in size like the Thr-927 → Ile mutant (Figure 4, pulse).

Within 9 h of the chase, the wild-type precursor was converted into the 95 kDa intermediate and the 76 kDa mature form of lysosomal α-glucosidase (Figure 4, chase 9 h). Also the Val-816 → Ile precursor was normally processed to 95 and 76 kDa, confirming our earlier conclusion that the replacement of Val by Ile is a polymorphism. The 108 kDa Thr-927 → Ile mutant precursor was converted into a 93 kDa intermediate whereafter a 76 kDa mature species of normal size was formed. This processing confirms our conclusions in the preceding paper that the glycosylation site at Asn-925 is located in a C-terminal propeptide that is cleaved off when the 95 kDa intermediate matures to 76 kDa. When Thr-927 was replaced by Ile the rate of maturation from 95 kDa to 76 kDa seemed slightly increased. The same effect was observed when this glycosylation site was deleted by an Asn-925 → Gln substitution (Hermans et al., 1993). The proteolytic processing of the precursor containing the Asp-645 → Glu substitution was delayed as was the processing of the double mutant precursor. (The respective 110 kDa and 108 kDa precursor species were still visible after 9 h of chase.)

A comparable picture was obtained after 30 h of chase, except that the Glu-545 and double mutant precursor were no longer detectable (Figure 4, chase 30 h). The mutant species are apparently degraded since they are not converted into mature enzyme and not secreted either (Figure 4, secretion).

The biosynthesis of lysosomal α-glucosidase containing the Arg-854 → Stop mutation is illustrated in Figure 5 (leucine incorporation). In a 6 h pulse period a truncated precursor of 98 kDa was formed. This 98 kDa species is not synthesized in cell line GM1935 since it is derived from the one allele that is not expressed.

Labelling with [^{32}P]P_i was performed to determine whether it was the single Asp-645 → Glu, the single Thr-927 → Ile or the combination of both mutations that interfered with phosphorylation (Figures 5 and 6). The incorporation of phosphate into β-hexosaminidase was taken as a reference. Phosphorylation of all mutant precursors was detectable but the degree of phosphate incorporation was low for the Asp-645 → Glu and the double mutant. It is evident that the phosphorylation defect is caused by the Asp-645 → Glu substitution rather than by the loss of the glycosylation site at Asn-925. Figure 5 shows that the truncated lysosomal α-glucosidase species also failed to incorporate phosphate. In cell line GM1935, the phosphorylation defect seems to be complete in contrast with that observed in the transfected COS cells. However, the expression of lysosomal α-glucosidase in the transient transfection system was much higher than in fibroblasts. Therefore it could very well be that phosphorylation of the lysosomal α-glucosidase precursor also occurs in cell line GM1935 but remains undetectable.

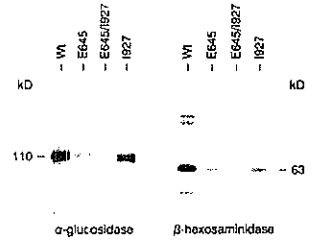

Figure 6 Labelling of lysosomal α-glucosidase and β-hexosaminidase with [^{32}P]P_i in COS cells transfected with wild-type or mutant lysosomal α-glucosidase species

COS cells were labelled for 3 h with [^{32}P]P_i and harvested directly. Lysosomal α-glucosidase and β-hexosaminidase were immunoprecipitated from the cell homogenates and analysed by SDS/PAGE. The molecular masses (kDa) of wild-type and mutant lysosomal α-glucosidase species are indicated.

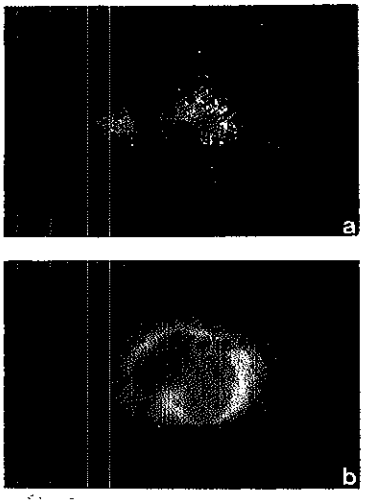

Figure 7 Localization of mutant lysosomal α-glucosidase in transfected COS cells studied by light microscopy

Transiently transfected COS cells were fixed and incubated with a rabbit polyclonal antiserum against human lysosomal α-glucosidase. Immune complexes were visualized with goat anti-rabbit IgG conjugated to fluorescein. (a) Thr-927 → Ile; (b) Asp-645 → Glu.

Summarizing the results presented in Table 2 and Figures 4–6, we conclude that it is the double mutant that mimics the molecular defect in cell line GM1936, but the deleterious effect on enzyme biosynthesis and function is caused by the Asp-645 → Glu rather than the Thr-927 → Ile substitution.

Subcellular localization

Immunocytochemistry was performed to study the intracellular localization of the mutant proteins in the transiently transfected COS cells. Typical punctate lysosomal labelling was obtained for

Table 3 Quantitative data on the lysosomal α-glucosidase labelling of the lysosomes in COS cells transiently transfected with wild-type and mutant cDNA constructs

The units for lysosomal labelling are the number of gold particles per lysosome ($n = 31$). Results are means ± S.E.M.

Type of mutation	Lysosomal labelling
Wild-type	43 ± 21
Thr-927 → Ile	31 ± 32
Asp-645 → Glu	13 ± 16
Double mutant	3 ± 3

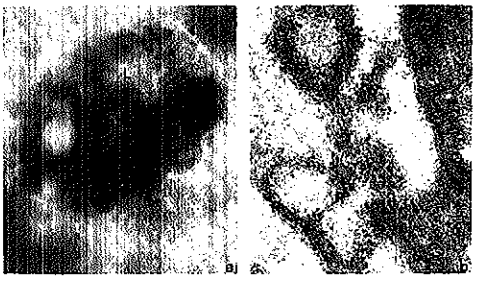

Figure 8 Subcellular localization of the Asp-645 → Glu mutant protein in transfected COS cells studied by immunoelectron microscopy

Ultrathin cryosections were incubated with rabbit polyclonal antiserum against human lysosomal α-glucosidase and subsequently with goat anti-rabbit IgG coupled to colloidal gold. Mutant protein was found in normal amounts in the endoplasmic reticulum (R) but very little enzyme was detectable in the lysosomes (L). M: mitochondrion. Bar is 0.1 μm. (a) Lysosome; (b) endoplasmic reticulum.

lysosomal α-glucosidase with the Thr-927 → Ile substitution (Figure 7a), but expression of lysosomal α-glucosidase with the single Asp-645 → Glu or the double mutation resulted in a labelling pattern characteristic of endoplasmic reticulum. Lysosomal labelling was barely detectable (Figure 7b). Semiquantitative data on the lysosomal labelling were obtained by immunoelectron microscopy on ultrathin cryosections. To this end, the number of gold particles per lysosome was counted in 31 randomly chosen lysosomes. The results are presented in Table 3. The amount of label in the lysosomes was not significantly different in cells expressing the wild-type lysosomal α-glucosidase compared with cells expressing lysosomal α-glucosidase with the Thr → Ile substitution at position 927. However, lysosomal labelling was significantly lower when Asp-645 was replaced by Glu or when the Asp-645 → Glu and the Thr-927 → Ile substitutions were present simultaneously. Both the wild-type and the Thr-927 → Ile-substituted lysosomal α-glucosidases were not only present in the lysosomes but also in the various compartments of the glycoprotein-transport pathway, including the endoplasmic reticulum, the Golgi cisternae and the trans-Golgi network (results not shown). The Asp-645 → Glu and the double-mutant α-glucosidase precursors were mainly localized in the endoplasmic reticulum (Figure 8). This abnormal enzyme distribution suggests that the mutant precursor is trans-

ported inefficiently from the endoplasmic reticulum to the Golgi. This is in line with the observed phosphorylation defect. The *N*-acetylglucosaminyl phosphotransferase responsible for the incorporation of ^{32}P in the lysosomal α-glucosidase precursor is thought to be located in the cis-most Golgi cisternae (Lazzarino and Gabel, 1988, 1989). Inefficient transfer of the mutant precursor to this compartment will therefore automatically lead to an apparent phosphorylation defect. On the basis of our present findings, we consider it less likely that the mutation in this patient affects the *N*-acetylglucosaminyl phosphotransferase-recognition site directly. The dramatic effect of the Asp to Glu substitution at position 645 is possibly caused by the change in secondary structure, as predicted by the Chou–Fasman algorithm. There are several examples in the literature of mutant proteins that do not exit the endoplasmic reticulum because of wrong folding (Pelham, 1989).

We have no explanation for the apparent discrepancy between our results and those reported by Martiniuk et al. (1991), where it concerns the effect of the amino acid substitutions. The experimental approach is only different in the sense that Martiniuk et al. (1991) used SV40-immortalized fibroblasts from a patient with GSDII for transient expression of lysosomal α-glucosidase cDNA whereas we used COS-1 cells. We have, however, not experienced a difference in the biosynthesis, the post-translational modification and the intracellular location of wild-type and mutant forms of lysosomal α-glucosidase in transfected COS-1 cells compared with human fibroblasts [compare Figures 1(a) and 4, and Hoefsloot et al. (1990b), Hermans et al. (1991a)]. Our conclusions are not solely based on the assay of lysosomal α-glucosidase activity but we have demonstrated in addition that the Asp-645 → Glu substitution accounts for delayed intracellular transport and deficient phosphorylation and proteolytic processing. The notion of Martiniuk et al. (1991) that the secondary structure of lysosomal α-glucosidase is not affected by the Asp-645 → Glu mutation is not in accordance with the Chou–Fasman analysis performed by us.

The most difficult to explain is the clinical phenotype of the patient. The mutation analysis has shown that only one allele is expressed and that this allele does not produce a substantial amount of functional lysosomal α-glucosidase. The results are in this respect no different from those obtained with fibroblasts from the patient. The low level of residual activity is typical for patients with an infantile form of glycogenosis type II. The relatively slow progression of the disease in this black adult patient remains an enigma, unless the biosynthesis of this mutant lysosomal α-glucosidase is different in muscle from that in fibroblasts or transfected COS cells.

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CHAPTER 6

THE LOSS OF A POLYMORPHIC GLYCOSYLATION SITE CAUSED BY THR-927 → ILE IS LINKED TO A SECOND POLYMORPHIC VAL-816 → ILE SUBSTITUTION IN LYSOSOMAL α-GLUCOSIDASE OF AMERICAN BLACKS.

(Adapted from Genomics, in press as a Brief Report)

THE LOSS OF A POLYMORPHIC GLYCOSYLATION SITE CAUSED BY THR-927 → ILE IS LINKED TO A SECOND POLYMORPHIC VAL-816 → ILE SUBSTITUTION IN LYSOSOMAL α-GLUCOSIDASE OF AMERICAN BLACKS

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Summary. DNA of 17 healthy, unrelated, American black individuals was screened for the occurrence of two amino acid polymorphisms which were found recently in an American black patient with glycogenosis type II (GSDII). One polymorphism, a Thr-927 → Ile substitution deleting the most carboxyl-terminal glycosylation site of lysosomal α-glucosidase, was found in this population with a frequency of 0.21. The second polymorphism being a Val to Ile substitution at position 816 was found with a frequency of 0.18 and was linked to the Thr-927 → Ile mutation in the same allele. The Asp-645 → Glu substitution, responsible for the lysosomal α-glucosidase deficiency in the American black patient was not detected amongst the 17 healthy individuals.

Inherited deficiency of the lysosomal hydrolase α-glucosidase (acid maltase) causes the lysosomal accumulation of glycogen, a condition known as glycogen storage disease type II (GSD II)(McKusick, 1990). The clinical phenotype is heterogeneous, and heterogeneity also exists at the molecular level (Reuser *et al.*, 1985). Point mutations were recently identified in two cases of severe infantile and one case of adult glycogenosis type II (Hermans *et al.*, 1991; 1993a; Zhong *et al.*, 1991; Martiniuk *et al.*, 1991). Besides mutations with a deleterious effect on enzyme function, several polymorphisms have been documented. Interesting are those polymorphisms that relate to racial differences, but they can pose a problem when mutant alleles are being analyzed. An example for this was an American black patient with an adult form of GSD II (GM1935). Mutation analysis revealed three amino acid substitutions that were not encountered in the Caucasian population. The three substitutions were Asp-645 → Glu (exon 14), Val-816 → Ile (exon 17) and Thr-927 → Ile (exon 19), and they were linked to the same allele (Martiniuk *et al.*, 1991; Hermans *et al.*, 1993a). The Thr-927 → Ile substitution deleting the most carboxyl-terminal N-linked glycosylation site was found to

have no effect on enzyme function (Martiniuk *et al.*, 1991; Hermans *et al.*, 1993a) in agreement with our studies on the role of the individual glycosylation sites (Hermans *et al.*, 1993b). There were different opinions, however, with respect to the effect of the other two substitutions. The Val-816 → Ile substitution was reported by Martiniuk et al. (1991) to cause the lysosomal α-glucosidase deficiency, but we concluded on the basis of more extensive studies that the Asp-545 → Glu substitution was actually the deleterious mutation and the Val-816 → Ile substitution a polymorphism (Hermans *et al.*, 1993).

In this report we have determined the incidence of the Val-816 → Ile and the Thr-927 → Ile substitutions among 17 healthy, unrelated American blacks. DNA was extracted from white blood cells or cultured fibroblasts. The exons 16-17 and 18-19 were PCR amplified, and allele specific oligonucleotide (ASO) hybridization was performed as described (Hermans *et al.*, 1993a). Cell line GM1935 was used as a positive control. The PCR primers were 18.2 (CCCATCCCATTCATCACCCG), 19.2 (AGAAGCCCACCTGG CCTCTC), 23 (AGGACGCCCAGCACCTTCTG) and 24 (TGAGAATTCCTCCACCAG GGTGGGGATGA) (Figure 1). ASO hybridization was performed with the following probes: Val-816 (ACACCATCAACGTCCACCTCC), Ile-816 (ACACCATCAACATCCA CCTCC), Thr-927 (CAACTTCACCTACAGCCCCGA) and Ile-927 (TCCAACTTCATC

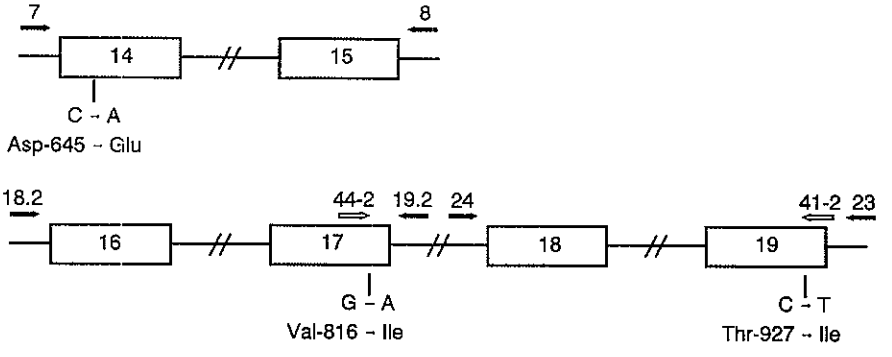

Figure 1: Schematic representation of the human lysosomal α-glucosidase gene.
The boxes represent exon 14 - 19. The arrows above the gene represent the various primers used. Solid arrows symbolize intron primers and open arrows show primers in which the terminal 3'-nucleotide is allele specific.

TACAGCCCC). Of the 17 individuals 10 were homozygous Thr-927/Thr-927, and 7 were heterozygous Thr-927/Ile-927. All of the first 10 individuals and 1 of the latter seven were homozygous Val-816/Val-816, whereas the remaining 6 individuals were heterozygous Val-816/Ile-816. The frequency of Thr-927 amongst the 17 individuals was 0.79. Ile -927 occurred with a frequency of 0.21. The frequency of Val-816 and of Ile-816 was 0.82 and 0.18, respectively (Figure 2).

The 17 DNA samples were also screened for the occurrence of the Asp-645 → Glu substitution (exon 14), associated with adult GSDII (GM1935). This substitution is caused by a C → A transition which abolishes an AatII restriction site (Martiniuk *et al.*, 1991). The AatII digestion was done on a PCR fragment spanning exons 14 and 15 obtained by using the primers 7 (TCACTAGGCCTGAGCTGGCTC) and 8 (CACTCAGCGGGATCC TGCTG) (Figure 1). Figure 3 shows that the AatII restriction site is missing in one of the two alleles of patient GM1935. Loss of the AatII site was not observed in any of the 17 individuals indicating that none of them carried the Asp-645 → Glu substitution. This

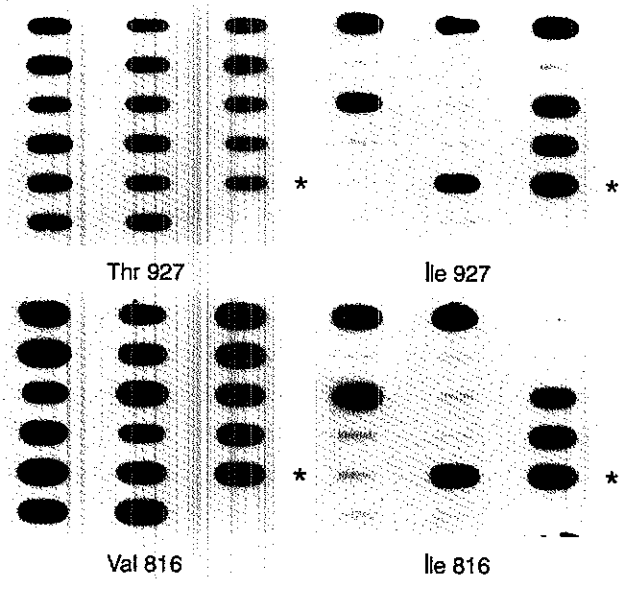

Figure 2: Allele specific oligonucleotide hybridization of PCR amplified DNA of American blacks. PCR products were denatured, applied on Hybond N^+ membranes via a slot blot apparatus and hybridized with [^{32}P]-labelled allele specific probes as indicated below the panels. DNA from cell line GM1935 is indicated with an asterisk. DNA in the remaining slots represent 16 of the 17 healthy individuals.

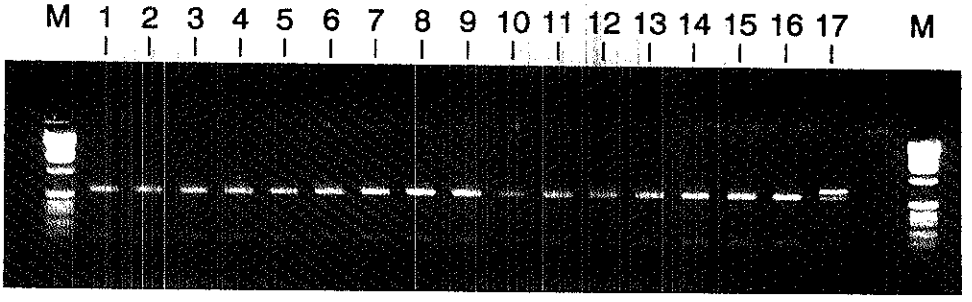

Figure 3: Analysis of the Asp-645 → Glu substitution by AatII digestion.
Exons 14 - 15 were PCR amplified, the fragments were digested with AatII and applied onto a 1.5% agarose gel. Lanes 1-16: healthy American blacks, lane 17: patient GM1935, lanes M: lambda DNA 1 kb marker (Gibco BRL).

sustains our previous conclusion that the Asp-645 → Glu substitution is responsible for the lysosomal α-glucosidase deficiency in GM1935. If the Val-816 → Ile substitution would have been associated with GSDII, as suggested (Martiniuk *et al.*, 1991), the prevalence of the disease amongst American blacks would have been 1 in 31 which is far higher than the estimated incidence (1:100.000)(Hers, 1989).

To investigate whether the Val-816 → Ile and the Thr-927 → Ile substitutions observed in 6 individuals were associated with the same allele as in cell line GM1935, we applied the "amplification refractory mutation system" (ARMS) of Newton et al. (1989).

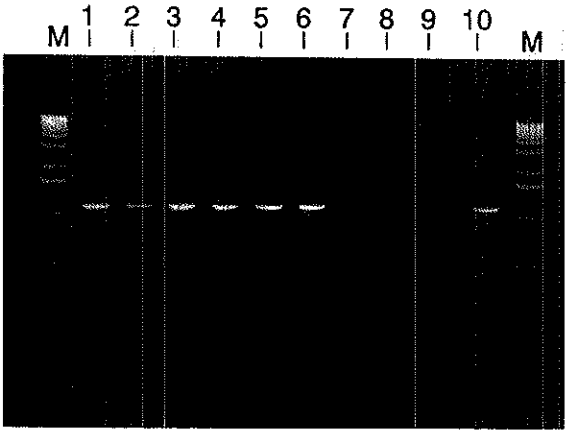

Figure 4: Specific amplification (ARMS) of the Ile-816/Ile-927 allele.
An aliquot of the PCR amplification reaction was applied directly onto a 1% agarose gel. Lanes 1-6: carriers of both the Ile-816 and the Ile-927 substitutions, lanes 7-8: individuals homozygous for the Val-816/Thr-927 allele, lane 9: carrier with only the Ile-927 substitution, lane 10: patient GM1935, lanes M: lambda DNA 1 kb marker (Gibco BRL).

ARMS has been designed to allow the rapid detection of point mutations via PCR amplification. It uses a PCR primer in which the terminal 3'-nucleotide is allele specific. Only if the primer matches the desired allele at the 3'-end an amplified product is produced, while a mismatched allele is not detected. A sense primer was designed (44-2) with the 3'-nucleotide matching the Ile-816 sequence (CCCCCTGGACACCATCAACA) and an antisense primer (41-2) with the 3'-nucleotide matching the Ile-927 sequence (CTTGGTGTCGGGGCTGTAGA)(Figure 1). PCR amplification can only occur if Ile-816 and Ile-927 are encoded by the same allele. Figure 4 shows that the expected PCR fragment of 1380 bp was only obtained with DNA from the six individuals carrying both substitutions and of cell line GM1935 and proves that the substitutions were linked. The association of the Asp-645 → Glu mutation with the relatively rare Ile-816/Ile-927 in stead of the more common Val-816/Thr-927 allele suggests that a linkage disequilibrium may exist. This can be tested with additional black GSDII patients.

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CHAPTER 7

GENOTYPE-PHENOTYPE CORRELATION IN A JUVENILE AND ADULT CASE OF GLYCOGEN STORAGE DISEASE TYPE II.

(Manuscript submitted)

GENOTYPE-PHENOTYPE CORRELATION IN A JUVENILE AND ADULT CASE OF GLYCOGEN STORAGE DISEASE TYPE II

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Summary. Glycogen storage disease type II (GSDII, M. Pompe) is caused by an inherited deficiency of the lysosomal enzyme α-glucosidase. In this paper we report on the identification of the mutant alleles of two unrelated Dutch patients, one with a juvenile and the other with an adult form of the disease.
Sequence analysis revealed that the juvenile patient was a genetic compound. One lysosomal α-glucosidase allele was characterized by a deleterious C to T transition at position 1644 causing the substitution of Pro-545 by Leu in lysosomal α-glucosidase. The second allele of this patient had a characteristic one base pair deletion at position 525 (ΔT-525) introducing a premature stop codon at position 660-662. The adult patient was shown to have two copies of the T-1644 allele.
The mutations were introduced in wild type cDNA and expressed in COS-1 cells to analyze their effect. Expression of the ΔT-525 allele did not result in the formation of an immunologically detectable polypeptide whereas the Pro-545 → Leu substitution allowed normal synthesis. The substitution did cause, however, a delay in the maturation of the lysosomal α-glucosidase precursor and did result in a 92% net reduction of enzyme activity. The residual lysosomal α-glucosidase activity of the adult patient was twice as high as that of the juvenile patient as predicted by the different allelic constitution of the two patients. The results are strongly suggestive for a direct correlation of genotype and phenotype.

INTRODUCTION

Glycogen storage disease type II (GSDII; M. Pompe) was the first lysosomal storage disorder recognized as such by the discovery of acid maltase (lysosomal α-glucosidase; EC. 3.2.1.3) deficiency in liver, heart and skeletal muscle of affected individuals (1). The ensuing search for lysosomal enzyme deficiencies in pathologically related diseases has resulted over the past three decades in the identification of the primary enzyme defect in more than 30 other lysosomal storage diseases (2). GSDII is an autosomal recessive trait with a broad clinical spectrum (3). At the one side of this

spectrum there are patients presenting at birth with massive accumulation of glycogen in muscle, heart and liver, and with a life expectancy of less than 2 years. Cardiorespiratory insufficiency is the major cause of death in this condition which is usually referred to as infantile GSDII (4). At the opposite side of the spectrum there are patients who are free of clinical symptoms for most of their life but who develop finally a slowly progressive myopathy. The first manifestation is often a weakness of the limb and girdle muscles, but patients may also present first with respiratory insufficiency (5). An extreme example of this so called adult form of GSDII is the patient described recently by Chancellor *et al.* (6). This patient was first diagnosed at the age of 68.

The molecular cause of clinical diversity in GSDII is not fully understood but there is a clear correlation between the progression of the disease and the extent of glycogen storage. The latter parameter seems to correlate inversely with the level of residual lysosomal α-glucosidase activity (7-12). But this correlation is not strict and exceptions have been encountered (9, 13). The exploration of the genetic basis for phenotypic variation has started ever since the lysosomal α-glucosidase cDNA and genomic sequences were cloned (14-16). The first reports on mutation analysis have appeared but the information is still limited compared to the published list of mutations that occur in the more commonly studied lysosomal storage diseases such as Gaucher disease and Tay-Sachs disease (17-22). It has nevertheless become clear that GSDII is not only at the protein level but also at the DNA level an extremely heterogeneous disease (9, 11-14, 16, 23-27). A further inventory of the mutations in the lysosomal α-glucosidase alleles of patients with distinct clinical phenotypes will help to evaluate the contribution of allelic diversity towards the clinical heterogeneity as opposed to variations in the genetic background and the possible influence of epigenetic factors. This type of information is potentially useful when therapeutic intervention is going to be undertaken in future.

In this study we have analyzed the lysosomal α-glucosidase genotype of two Dutch patients, one with a juvenile and the other with an adult form of GSDII. The two cases were studied in combination for reason that a normal synthesis but a defective maturation of lysosomal α-glucosidase had been observed in fibroblasts of both patients. The residual enzyme activity in the cells amounted to approximately 1.5% for the juvenile and 3% for the adult patient. These activities were considered to be exceptionally low in comparison with those of other patients with a similar clinical phenotype (9, 25).

The genetic analysis described in this paper reveals that the two patients share a mutant allele which has the potential capacity to produce lysosomal α-glucosidase activity up to 8% of the normal level. The adult patient has two copies of this allele, the juvenile patient only one. Since the second mutant allele of the latter patient is functionally silent, these data present a good example of a genotype-phenotype correlation in GSDII.

MATERIALS AND METHODS

Case reports. Fibroblasts cell line 84RD390 was derived from an adult female with signs of glycogen storage disease type II. The patient was 42 years old when she was diagnosed. Her complaints were that she had difficulty in climbing stairs. Her pelvic muscle and muscles of the proximal upper and lower limbs were found to be weakened. Motor conduction velocities were normal. The level of serum creatine kinase was increased till 540 U/L (110 U/L is normal). A muscle biopsy was taken from the biceps, and considerable variation in fiber size was noted. Vacuoles with PAS-positive material were present in many of the fibers and the acid phosphatase activity was increased. The glycogen content of the muscle (78 μg/mg protein) was within the normal range (30 - 120 μg/mg protein). The lysosomal α-glucosidase activity was decreased in the muscle biopsy species, in the leucocytes and in cultured fibroblasts of the patient. The patient is at present 50 years old.

Fibroblast cell line 124LAD was derived from a juvenile female who was examined at the age of 13 years. She had a progressive weakness of the proximal limb muscles. At the age of 6 years she was still able to walk and swim. Neurological examination revealed a severe weakness of the neck flexors and tensors as well as of the scapular and limb girdle muscles. The scapular muscles were atrophic. Little atrophy was noted of the lower limbs. There was no hypertrophy. Reflexes were symmetrically normal. The serum creatine kinase was elevated (231 U/L). A muscle biopsy was taken from the left quadriceps femoris, and most fibers had an abnormal structure. One or more vacuoles of varying size were seen in cross sections and some fibers were necrotic. The material contained in the vacuoles was PAS-positive, and all vacuoles showed a strong activity of acid phosphatase. The vacuoles were identified as being lysosomes by immuno-electron microscopy. The lysosomal α-glucosidase activity in the muscle biopsy specimen, in leucocytes and fibroblasts of the patient was severely deficient. The patient died at the age of 18 years.

DNA amplification and sequencing. DNA and RNA were extracted from cultured fibroblasts following standard procedures (28). PCR amplification using intronic primers specific for lysosomal α-glucosidase was as described (17). DNA fragments were sequenced using the T7 polymerase sequencing kit (Pharmacia LKB Biotechnology Inc.) with appropriate primers.

Site directed mutagenesis. Site directed mutagenesis was carried out as described before (29). The following oligonucleotides, synthesized on an Applied Biosystems 381A DNA synthesizer were used: Leu-545 CCCTACGTGCTTGGGGTGGTT and ΔT-525 ATGATG GAGACGAGAACCGCC.

Transient expression in COS-1 cells. COS-1 cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics, at 37 °C. The transfection protocol was as described before (30). The cells were harvested at 90 h after transfection. Cell homogenates were prepared by repeated freezing and thawing of cell pellets in distilled water. The activity of lysosomal α-glucosidase in the cell homogenates was measured with 4-methylumbelliferyl-α-D-glucopyranoside (4-MU) or with glycogen as substrate (29).
To study the synthesis and post translational modification of lysosomal α-glucosidase, COS-1 cells were labelled for 2 h with [^{3}H]-leucine (190 μCi/mmole)(Amersham U.K.) at 65 h after transfection, and lysosomal α-glucosidase was immunoprecipitated either directly (pulse) or after 16 h of chase. The different molecular species of lysosomal α-glucosidase were separated by SDS-PAGE as described (25).
The intracellular location of the biosynthetic forms of lysosomal α-glucosidase in the transfected COS-1 cells was established with the use of immunocytochemical procedures as described before (30).
Southern blotting. Restriction enzyme digests were performed on 10-20 μg of DNA in the appropriate buffers. DNA fragments were separated on 0.7% (w/v) agarose gels and subsequently blotted onto Zeta-Probe blotting membranes (Biorad). Filters were hybridized with lysosomal α-glucosidase cDNA using standard protocols (28).
Fluorescent *In situ* hybridisation (FISH). Metaphase spreads were obtained from peripheral blood lymphocytes following standard techniques (31). *In situ* hybridisation was performed essentially as described by Van Hemel *et al.*, 1992 (32). A centromere specific alphoid DNA probe p17H8 (33) was used for the identification of chromosome 17. For the labelling of the lysosomal α-glucosidase gene we used the three genomic Bgl II fragments of 14, 10.5 and 8.5 kb cloned in plasmid pTZ19, which represent the complete gene (15). The probes were labelled by nicktranslation using the BioNick system (Gibco BRL). The hybridization mixture contained 30 ng of each of the α-glucosidase probes, 4 ng of p17H8 and 1 μg of Lot-1 DNA (Gibco BRL) as competitor. The probes were dissolved in 10 μl of 50% deionized formamide, 10% dextran sulphate, 1% Tween in 2xSSC, pH 7.0. The probes and the chromosome spreads were denatured separately. Reassociation of repeat sequences was allowed for 1 h at 37 °C, whereafter 10 μl of the probe mixture was applied to the chromosome spreads. Hybridization was performed overnight at 37 °C.
Immunocytochemical detection of the probes was achieved by successive incubation with fluorescein conjugated avidin (avidin-FITC; Vector. LAB.), biotinylated goat anti avidin and again avidin-FITC. Slides were mounted in medium containing propidium iodide for counterstaining. The chromosome spreads were examined under a Leitz Aristoplan microscope.

RESULTS

DNA sequence analysis was performed to identify the mutations in the lysosomal α-glucosidase gene (GAA) of two unrelated Dutch patients, one with a juvenile (124LAD)

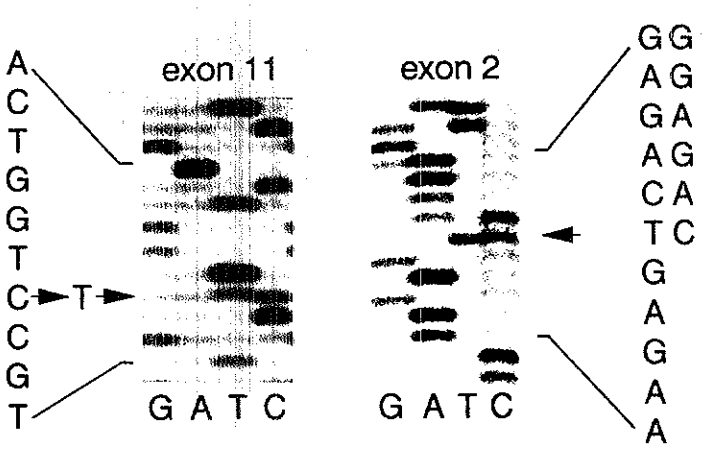

Figure 1: Mutations in the genomic sequence of the lysosomal α-glucosidase gene of patient 124LAD with juvenile GSDII.
Sequence analyses were performed directly on PCR amplified genomic DNA. The deletion of a single T at position 525 in exon 2 and the C to T transition at position 1644 in exon 11 are indicated with arrows. The wild type sequence occurs along with the mutant sequence at both marked positions demonstrating that 124LAD is heterozygous for both mutations.

and the other (84RD390) with an adult form of GSDII. The DNA was isolated from cultured fibroblasts, and the exons of the gene were PCR amplified and sequenced directly. One point mutation was discovered in exon 11 and a second in exon 2 of the GAA gene of the more severely affected juvenile patient (124LAD). The mutation in exon 11 concerned a C to T transition at position 1644[1] resulting in the substitution of Pro-545 by Leu. The mutation in exon 2 was a one base pair deletion at position 525 (ΔT-525) introducing a premature stop codon at position 660-662. The patient was heterozygous for both mutations since the normal lysosomal α-glucosidase sequence occurred along with the mutant sequence at each of the two positions (Figure 1). The ΔT-525 and the C-1644 → T mutation were considered to represent each a different mutant allele.

To study the effect of both these mutations on the biosynthesis and function of lysosomal α-glucosidase they were introduced in the wild type cDNA by site directed mutagenesis and expressed in monkey kidney cells (COS-1). Two independent clones of each mutant construct were tested. The COS-1 cells were harvested 90 h after transfection and the activity of lysosomal α-glucosidase was assayed using glycogen as the natural and

[1]counting the A of the initiation codon as position 1

Table 1: Catalytic activities of lysosomal α-glucosidase mutants in transiently transfected COS-1 cells 90 h after transfection.

Type of mutation	Catalytic activity	
	4-MU[a]	Glycogen[b]
COS-1 cells	39.5 ± 4.2 [c]	286 ± 33
Wild type	357.5 ± 30.0	4951 ± 481
ΔT-525	37.6 ± 1.8	252 ± 19
Pro-545 → Leu	67.8 ± 4.8	641 ± 27

The activity is expressed as [a] nmol 4-MU/ mg protein/ h or as [b] nmol glucose/ mg protein/ h.
[c] Indicated is average ± SEM of 4 assays.

4-methylumbelliferyl α-D-glucopyranoside as an artificial substrate (Table 1). To have a negative control COS-1 cells were transfected with E. coli ß-galactosidase cDNA. Expression of the wild type lysosomal α-glucosidase cDNA in COS-1 cells resulted in a 10 to 20 fold increase of enzyme activity compared to the negative control, whereas only a 2 to 3 fold increase of activity, depending on the substrate used, was measured upon expression of lysosomal α-glucosidase containing the Pro-545 → Leu substitution. No gain of activity was measured in COS-1 cells transfected with the ΔT-525 mutant construct.

To investigate the molecular nature of the enzyme deficiency, we followed the synthesis and maturation of the mutant lysosomal α-glucosidase species. The results of a pulse-chase labelling study performed at 60 h after transfection are illustrated in Figure 2. After two hours of [^{3}H]-leucine incorporation (pulse) a normal synthesis of the 110 kD precursor of lysosomal α-glucosidase was registered in the COS-1 cells transfected with either the wild type cDNA or the mutant cDNA encoding Leu-545. The truncated lysosomal α-glucosidase precursor encoded by the ΔT-525 cDNA was not detectable. After 16 h (chase) the usual 95 kD processing intermediate and the 76 kD mature enzyme had formed from the wild type precursor. In contrast, the precursor containing the Pro-545 → Leu mutation was still present and only minute amounts of the 95 kD and 76 kD species had formed. These results point to a delay in transport of the mutant precursor to the prelysosomal and lysosomal compartments where the proteolytic maturation is known to occur (34).

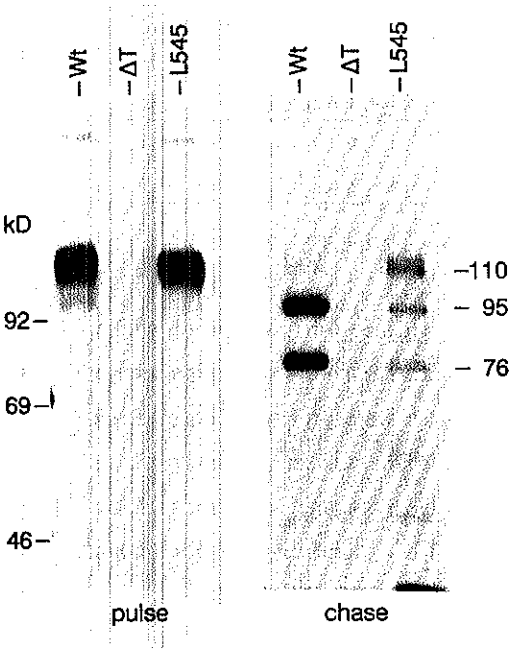

Figure 2: Synthesis and processing of wild type and mutant lysosomal α-glucosidase species in transiently transfected COS-1 cells.
COS-1 cells transfected with wild type or mutant cDNA constructs were labelled for 2 h with [^{3}H]-leucine and harvested directly (pulse) or after a subsequent period of 16 h (chase). Lysosomal α-glucosidase was immunoprecipitated from the cell homogenates and analyzed by SDS-PAGE. The molecular masses (kD) of wild type α-glucosidase species are indicated.

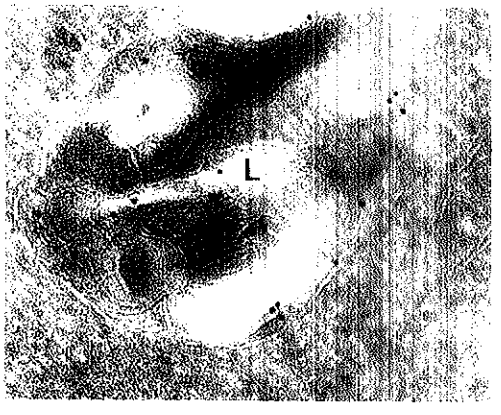

Figure 3: Lysosomal localization of the Pro-545 → Leu mutant protein in transfected COS-1 cells studied by immuno-electron microscopy.
Ultrathin cryosections were incubated with rabbit polyclonal antiserum against human lysosomal α-glucosidase and subsequently with goat anti-rabbit IgG coupled to colloidal gold. Magnification: 98000 x.

Table 2: Quantitative data on the lysosomal α-glucosidase content of the lysosomes in COS-1 cells transiently transfected with wild type and mutant cDNA constructs.

Type of mutation	lysosomal labelling[a]	SEM
Wild type	43	4
Pro-545 → Leu	22	5

[a] Average number of gold particles per lysosome (n=31)

To obtain supportive evidence for this view we studied the intracellular location of lysosomal α-glucosidase using immuno-electron microscopy. The wild type and Leu-545 mutant lysosomal α-glucosidase were present in equal amounts in the ER of transfected COS-1 cells but the mutant enzyme was quantitatively reduced in the Golgi and lysosomal compartments. A representative lysosome is shown in Figure 3. Semiquantitive data on the lysosomal labelling revealed a significantly lower amount of lysosomal α-glucosidase in cells transfected with the mutant compared to the wild type cDNA (Table 2).

The abnormalities in the biosynthesis, the transport and the function of the mutant lysosomal α-glucosidase species expressed in COS-1 cells mimic those reported to occur in cultured fibroblasts of patient 124LAD (25).

To our surprise the milder affected unrelated adult patient (84RD390) appeared to have the same Leu-545 mutant allele with the deleterious C → T transition at position 1644. But, there was no indication for this patient to be a genetic compound. On the contrary, the patient seemed to be homozygous for the Leu-545 allele (Figure 4). There was reason, however, to be suspicious since consanguinity was not known in the patients family. It was realized that a complete lack of the second allele or the deletion of exon 11 and surrounding intron sequences would have gone undetected in the PCR based method that was used to amplify the exons of the GAA gene. To check for the possible occurrence of a deletion we performed Southern blot analysis on genomic DNA from the patient. DNA from her healthy son was taken as a control. Figure 5A shows that the Hind III fragments of the patient are of the right size, while a deletion of exon 11 should have caused a change in the size of the 4.6 kb fragment. The existence of deletions did not become evident either from the Bgl II restriction fragment pattern covering also the complete GAA gene (Figure 5B). Finally, we performed *in situ* hybridization on metaphase chromosomes to demonstrate unequivocally the presence of the GAA alleles on

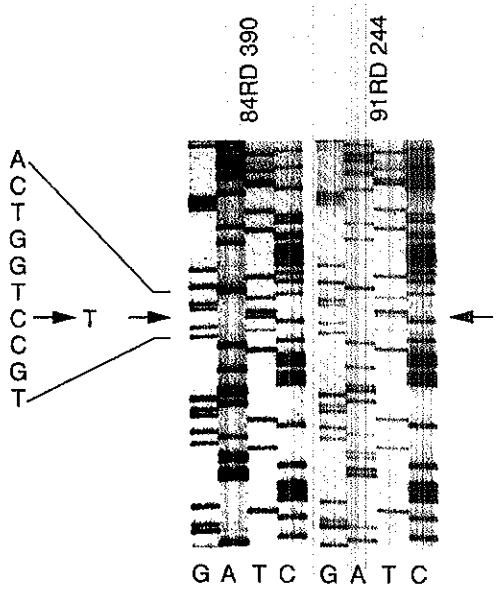

Figure 4: Mutation in the genomic sequence of the lysosomal α-glucosidase gene of patient 84RD390 with adult GSDII and in the gene of her healthy son 91RD244.
Sequence analyses were performed directly on PCR amplified genomic DNA. The C to T transition at position 1644 is indicated with an arrow. 84RD390 is homozygous for the C to T transition whereas 91RD244 is heterozygous.

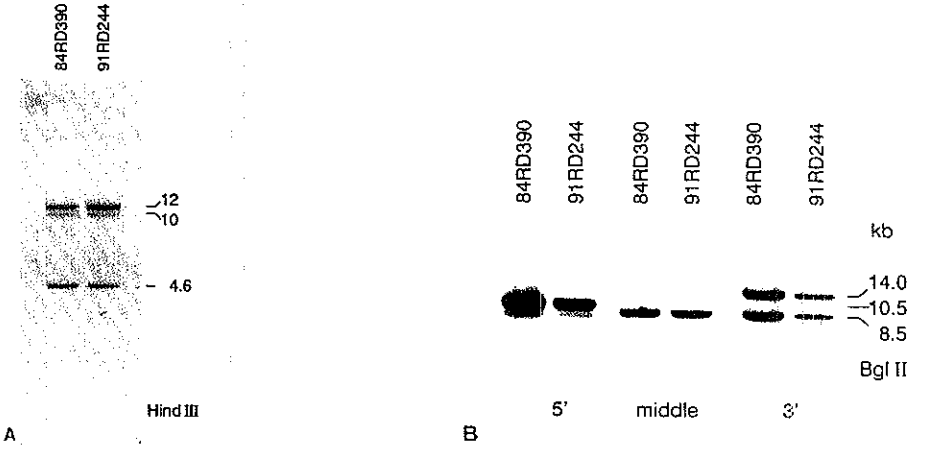

Figure 5: Southern blot analyses of genomic DNA from patient 84RD390 and her healthy son (91RD244).
Genomic DNA was digested with HindIII (A) or with BglII (B) and analyzed by Southern blotting using cDNA probes of lysosomal α-glucosidase covering the complete gene. The estimated length of the DNA fragments is given in kilo bases (kb).

both chromosomes 17. Cloned fragments of the gene were used as a probe. The results are illustrated in Figure 6. Chromosome 17 is easily recognized by the centromere staining. Fluorescent dots marking the location of the GAA gene were seen at the end of the long arm of both chromosomes 17. Using two highly polymorphic markers (VNTR probes, p144-D6 and pYNH37-3) of chromosome 17 (35, 36) we were able to demonstrate that the patient, homozygote for the Leu-545 allele, had not inherited both her chromosomes 17 from the same parent (Figure 7).

DISCUSSION

In this report we have identified the mutations in the lysosomal α-glucosidase alleles of two Dutch patients suffering from GSDII. The older patient with an onset of symptoms in the fourth decade of live was demonstrated to have two copies of a mutant allele with a Pro-545 → Leu causing C-1644 to T transition in exon 11 of the GAA gene.

The younger patient with onset of symptoms in the first decade of life was identified as a genetic compound. One of her alleles appeared to be the same as that of the adult patient, the other had a characteristic ΔT-525 mutation. The product of the ΔT-525 allele with a predicted size of 24 kD was not detectable upon transient expression in COS-1 cells. A polypeptide of this size was similarly lacking in cultured fibroblasts of the patient (25). The change of primary structure and conformation of the ΔT-525 protein could possibly lead to instability of the polypeptide or to loss of antigenicity. But it may also be that the mRNA containing the premature stop codon is unstable (37).

The Leu-545 precursor expressed in COS-1 cells was not distinguishable from the 110 kD wild type precursor when analyzed by SDS-PAGE. But the maturation of the mutant precursor was delayed and inefficient as it was in cultured fibroblasts of both patients (9, 25). As a net result the Pro-545 → Leu substitution limits the formation of mature 76 kD enzyme.

The delay in maturation of the Leu-545 precursor could be due to improper folding since the Chou & Fasman algorithms for secondary structure prediction show a disruption of a protein turn and a reduction of a ß-pleated sheet (38). As proper folding is believed to be a prerequisite for exit of proteins from the ER (39, 40) the mutation may

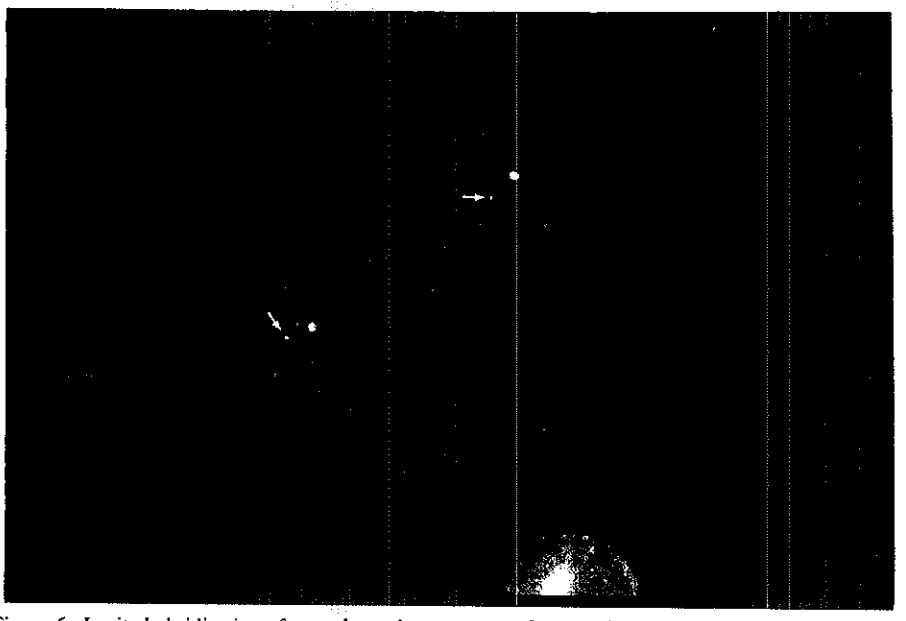

Figure 6: *In situ* hybridization of metaphase chromosomes of patient 84RD390.
Metaphase chromosomes were hybridized with a chromosome 17 centromere probe (p17H8) and with probes derived from the lysosomal α-glucosidase gene. The fluorescent dots marking the GAA gene are indicated with arrows.

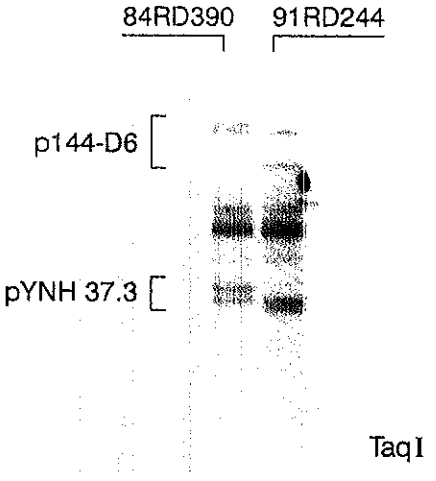

Figure 7: Identification of chromosomes 17 of patient 84RD390 and her healthy son (91RD244).
Genomic DNA was digested with TaqI and analyzed by Southern blotting using the VNTR probes p144-D6 and pYNH37-3 to identify the different chromosomes 17. Two fragments differing in size are revealed by both probes.

very well lead to the observed accumulation and degradation of the lysosomal α-glucosidase precursor. Obviously, a portion of the molecules escapes degradation, enters the Golgi complex and is finally delivered to the lysosomes, where proteolytic processing to 95 and 76 kD occurs (34). This lysosomal pool of enzyme is apparently active and accounts for the residual lysosomal α-glucosidase activity of 8% as measured in the transfected COS-1 cells.

Variability of the clinical phenotype is a hallmark of lysosomal storage diseases and can be ascribed in order of importance to i) allelic diversity, ii) variability of genetic background and iii) epigenetic factors. The contribution of the latter two factors is the most difficult to evaluate, but cannot be ignored. This is indicated by the fact that for instance in Gaucher disease the severity of clinical symptoms can vary considerably even when siblings carry the same Asn-370 → Ser mutation in both their glucocerebrosidase alleles (20, 41). The occurrence of allelic diversity has been documented in several of the more than thirty different lysosomal storage diseases and has given an insight in the genotype-phenotype correlation. Mutations causing gene disruption, splice defects or instability of mRNA lead predictably to complete loss of lysosomal enzyme function and are in homozygous form consistently associated with a severe form of disease. Several examples of these types of mutation have been reported (42-45). The effect of point mutations is in general more difficult to predict since point mutations can interfere with enzyme function as well as with posttranslational modification and transport. The most reliable parameter for predicting the clinical phenotype is, therefore, the level of residual enzyme activity. This has been demonstrated by now in a number of lysosomal storage diseases including GSDII. Patients with GSDII present consistently the most severe infantile phenotype when lysosomal α-glucosidase mRNA is undetectable. Patients with a normal synthesis of mRNA but with a complete deficiency of functional enzyme are also severely affected (11, 14, 16, 17, 19, 23). One of these patients from a consanguineous family was reported to have a Glu-521 → Lys substitution preventing the intracellular transport and maturation of the mutant lysosomal α-glucosidase precursor (17). Milder affected patients with later onset and slower progression of symptoms have virtually always a certain percentage of residual lysosomal α-glucosidase activity. This percentage varies mostly from 10 to 25% among patients with adult GSDII but can be as low as 3% in some exceptional cases to which 84RD390 belongs (9, 18).

We were fortunate to find in our studies a nice example of a genotype-phenotype correlation in GSDII. Patient 124LAD with a juvenile subtype and a residual lysosomal α-glucosidase activity of 1.5% in cultured fibroblasts was found to have two different mutant alleles. The one with the characteristic ΔT-525 mutation was shown to be functionally silent, the other (Leu-545) capable to maintain a low level of catalytically active lysosomal α-glucosidase. The adult patient (84RD390), earlier reported to have twice as much residual activity as patient 124LAD was demonstrated to have two copies of the Leu-545 allele. It, thus seems that gene dosage accounts fully for the difference in residual lysosomal α-glucosidase activity and the clinical phenotype of the two patients.

The data presented in this study may provide an explanation for the apparent discrepancy between the exceptionally low residual lysosomal α-glucosidase activity of patient 84RD390 and her relatively mild clinical phenotype. When expressed in COS-1 cells the Leu-545 mutant allele produces lysosomal α-glucosidase at 8% of the normal activity level which is more than twice as high as in *in vitro* cultured fibroblasts and muscle cells of the patient (9). This indicates that the mutant allele is not expressed at full capacity in the *in vitro* cultured cells of the patient. Recent results from our laboratory suggest that the maximal capacity is exploited, however, *in vivo* in storage prone muscle fibers of the patient (Willemsen, unpublished results).

The finding of the same Leu-545 mutant allele in both patients is remarkable since sporadic mutations predominate in rare genetic disorders like GSDII. The underlying cause may be a founder effect since both patients, although not known to be related, are of Dutch origin. Also the fact that patient 84RD390 was homozygous for the Leu-545 allele while there was no record of consanguinity in the family points in this direction. For this reason we have started to screen a larger collection of Dutch patients with the juvenile or adult form of GSDII for the possible occurrence of the Leu-545 mutant allele.

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CHAPTER 8

TWO MUTATIONS AFFECTING THE TRANSPORT AND MATURATION OF LYSOSOMAL α-GLUCOSIDASE IN AN ADULT CASE OF GLYCOGEN STORAGE DISEASE TYPE II.

(*Human mutation, in press*)

TWO MUTATIONS AFFECTING THE TRANSPORT AND MATURATION OF LYSOSOMAL α-GLUCOSIDASE IN AN ADULT CASE OF GLYCOGEN STORAGE DISEASE TYPE II

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Summary. The autosomal recessive glycogen storage disease type II is associated with a deficiency of lysosomal α-glucosidase (acid maltase). This paper reports on the mutations in the lysosomal α-glucosidase alleles of an adult patient. A G-1927 to A transition was discovered in exon 14 causing the substitution of Gly-643 by Arg and a second C-2173 to T transition in exon 15 resulting in the substitution of Arg-725 by Trp. Each of the mutations was located in a different allele. The mutations were introduced in the wild type lysosomal α-glucosidase cDNA and expressed in COS cells. Both mutations had a similar effect. The synthesis of the mutant enzyme precursors was not disturbed but the intracellular transport and maturation was impaired. As a result there was an overall deficiency of catalytic activity.

INTRODUCTION

Lysosomal α-glucosidase (acid maltase, EC 3.2.1.3) is an acid hydrolase required for the degradation of glycogen. Deficiency of this enzyme results in glycogen storage disease type II (GSDII), an autosomal recessive disorder (McKusick, 1990). The disease is clinically heterogeneous. When signs of cardiomegaly and generalized hypotonia present shortly after birth, the patients usually die within the first two years of life. In other cases, however, the disease may become manifest only after the patients have reached adulthood and the symptoms remain limited to skeletal muscle weakness (Engel, 1986). The variation in clinical phenotype is thought to relate to the occurrence of different mutations, each of which may have a distinct effect on the functional capacity of the mutant enzyme.

Indirect evidence for allelic variation in GSDII was obtained by studying the lysosomal α-glucosidase expression in fibroblasts and muscle cells of clinical variants

(Reuser et al., 1978, 1985, 1987; Beratis et al., 1978, 1983; Martiniuk et al., 1986, 1990a; Hoefsloot et al., 1988, 1990a; Van der Ploeg et al., 1988). Recent characterization of mutant alleles has provided direct prove of genetic heterogeneity (Hermans et al., 1991a, 1993; Zhong et al., 1991).

In this paper we describe the identification of point mutations in each of the lysosomal α-glucosidase alleles of a patient with an adult form of GSDII (Trend et al., 1985, Case I). The patient who was previously reported to have a very low residual enzyme activity of 1-2% (Trend et al., 1985; Reuser et al., 1987) is shown to be a genetic compound. A point mutation leading to an amino acid substitution was found in each of the two alleles. The mutations were introduced in the wild type lysosomal α-glucosidase cDNA by site directed mutagenesis and were proven to account for the molecular defect observed in the patients fibroblasts.

MATERIALS AND METHODS

DNA amplification and sequencing. DNA was extracted from cultured fibroblasts following standard procedures (Sambrook et al., 1989). PCR amplification using intronic primers specific for lysosomal α-glucosidase was as described (Hermans et al., 1991a). Each 100 μl PCR reaction contained approximately 0.5 μg of DNA, 100 pmol of each primer and 2 units of Taq polymerase (BRL) in the standard PCR buffer recommended by Sambrook et al. (1989). Amplification was performed in a Perkin-Elmer Cetus DNA amplifier. The first cycle was: 5 minutes denaturation at 94 °C, 5 minutes annealing at 59 °C and 5 minutes DNA synthesis at 72 °C. Subsequent cycles (25-30) consisted of 2 minutes at 94 °C, 2 minutes at 59 °C and 5 minutes at 72 °C. The DNA synthesis step of the final cycle was extended to 15 minutes. In some instances PCR amplified DNA fragments were cloned in a T-tailed Bluescript plasmid vector. This so called T-vector was constructed as described by Marchuk et al. (1991). DNA fragments were sequenced using the T7 polymerase sequencing kit (Pharmacia LKB Biotechnology Inc.) with appropriate primers.

Site directed mutagenesis. Site directed mutagenesis was carried out as described before (Hermans et al., 1991b). The following oligonucleotides, synthesized on an Applied Biosystems 381A DNA synthesizer, were used: Arg-643 GCCTCTGGTCAGGGCCGAC GT and Trp-725 GACCGTGGCCTGGCCCCTCTT

Transient expression in COS cells. Wild type and mutant cDNA constructs were cloned in the eukaryotic expression vector pSG5 (Green et al., 1988) and transfected to COS cells as described (Hoefsloot et al., 1990b). Lysosomal α-glucosidase activities in the cell homogenates and the culture medium were measured with 4-methylumbelliferyl-α-D-glucopyranoside (4-MU) as an artificial substrate and with glycogen as the natural substrate as described (Hermans et al., 1991b).

To study the biosynthesis and processing of lysosomal α-glucosidase, COS cells were

labelled for 2 h with Tran-[^{35}S]-methionine (ICN) or for 4 h with carrier-free [^{32}P]-phosphate (Amersham) at 60 h after transfection, and lysosomal α-glucosidase was immunoprecipitated using a polyclonal antiserum raised in rabbits against human placental α-glucosidase together with protein A-Sepharose beads (Pharmacia) either directly (pulse) or after a 7 or 30 h chase. SDS-PAGE analysis was performed as described (Hasilik and Neufeld, 1980a, 1980b).

RESULTS AND DISCUSSION

To identify the mutations responsible for the adult form of GSDII in this patient we decided to PCR amplify all the exons of the lysosomal α-glucosidase gene and to determine their sequence. Most PCR products were sequenced directly. In some instances, however, the PCR fragments were cloned prior to sequencing, and four separate clones were analyzed.

Seven base pair alterations were found in the coding region of the lysosomal α-glucosidase gene when compared with the wild type sequence published by Hoefsloot et al. (1988) (EMBL entry number Y00839) (Table 1). The patient was heterozygous for all

Table 1: Base pair substitutions (Comparison with the lysosomal α-glucosidase cDNA sequence in the EMBL/Genbank (Y00839).

nucleotide position[a]	exon	cDNA	patient	amino acid alteration	reference
Mutations					
1927	14	G	A	Gly-643 → Arg	
2173	15	C	T	Arg-725 → Trp	
Polymorphisms					
642	3	C	T	-	1
921	5	A	T	-	
1374	9	C	T	-	2
1581	11	A	G	-	1,2,3,4
2133	15	A	G	-	1

[a] Nucleotide position counted from the initiation codon of the precursor.
(1) Martiniuk et al., 1990b; (2) Hermans et al., 1993; (3) Hoefsloot et al., 1990c;
(4) Hermans et al., 1991a.

seven alterations, five of which were polymorphisms, not resulting in a change at the amino acid level. The A to T transition at position 921 had not been described before. Both the G-1927 → A transition in exon 14 and the C-2173 → T transition in exon 15 were marked as the potential disease causing mutations since they result in amino acid substitutions: Gly-643 → Arg and Arg-725 → Trp, respectively. To define whether both mutations were located in the same allele or in different alleles we PCR amplified the exons 14 and 15 as one fragment from genomic DNA and we cloned this fragment. Two clones were found to have the Gly-643 → Arg mutation in combination with the wild type sequence for Arg-725. Two other clones had the wild type sequence for Gly-643 but contained the Arg-725 → Trp mutation. From this we conclude that the patient is a genetic compound with one mutant allele characterized by the Gly-643 → Arg substitution and the other by the Arg-725 → Trp substitution. It is noteworthy that the parents of this patient are first cousins (Trend et al., 1985, Case I). Thus, this patient was more likely to have the same mutation in both alleles than to be a genetic compound.

To confirm that the Gly-643 → Arg and the Arg-725 → Trp mutations were responsible, indeed, for the lysosomal α-glucosidase deficiency of the patient, we introduced both mutations separately in the wild type lysosomal α-glucosidase cDNA by site directed mutagenesis. Two independent clones of each mutant construct were expressed transiently in COS cells and the activity of lysosomal α-glucosidase was measured at 96 h after transfection (Table 2). A transfection with lysosomal α-glucosidase

Table 2: Catalytic activities of lysosomal α-glucosidase mutants in transiently transfected COS cells 96 h after transfection.

Type of cDNA construct	Catalytic activity		
	Cells[a]	Medium[a]	Cells[b]
antisense	42.3 ± 3.6[c]	7.1 ± 0.4	115 ± 15
Wild type	663.6 ± 32.6	276.7 ± 10.9	4118 ± 201
Gly-643 → Arg	81.7 ± 2.1	5.0 ± 0.4	260 ± 8
Arg-725 → Trp	85.7 ± 1.3	6.6 ± 0.7	268 ± 5

The activity is expressed as [a] nmol 4-MU/ mg protein/ h or as [b] nmol glucose/ mg protein/ h. [c] Indicated is average ± SEM of 6 assays.

cDNA in antisense orientation was performed as a negative control. As shown in Table 2, both mutant enzymes were severely defective in their ability to hydrolyse 4-methylumbelliferyl-α-D-glucopyranoside as well as glycogen. The results are in accordance with the low level of residual lysosomal α-glucosidase activity in the patients fibroblasts (Trend et al., 1985; Reuser et al., 1987).

To identify the defect more precisely, we have studied the biosynthesis of lysosomal α-glucosidase. The enzyme is known to be synthesized as a precursor of 110 kD which is transported from the endoplasmic reticulum to the Trans Golgi Network. From this compartment the major part of precursor molecules is transported to the lysosomes and proteolytically converted to 76 kD and finally to 70 kD via a long lived intermediate of 95 kD. A minor part of precursor molecules is transported to the plasmamembrane and secreted. The secreted precursor as well as the 95 kD, 76 kD and 70 kD intracellular forms of lysosomal α-glucosidase are catalytically active (Wisselaar et al,, 1993). A pulse chase labelling was performed at 60 h after transfection to study the synthesis and fate of the mutant enzyme species. The results are illustrated in Figure 1. The 110 kD precursor of lysosomal α-glucosidase and the long lived 95 kD intermediate were detectable in COS cells transfected with both the wild type and the mutant cDNA's after a 2 h pulse. After 7 h of chase practically all wild type precursor molecules were converted to 95 kD and 76 kD species. In contrast, the Gly-643 → Arg or the Arg-725 → Trp precursor molecules were still present, and processed forms of lysosomal α-glucosidase were hardly detectable. After 30 h of chase the molecular pattern of the wild type species was very much the same as after 7 h. The mutant enzyme species, however, had disappeared almost completely. A further difference between the wild type and the mutant enzyme species was that only the wild type precursor was secreted into the culture medium. From the deficiency of lysosomal α-glucosidase in both the lysosomal and secretory pathway we infer that the biosynthesis derails before the precursor leaves the Trans Golgi Network from where these two pathways divert. Export from the endoplasmic reticulum is probably the limiting step since the phosphorylation of mannose residues, which occurs in the cis Golgi cisternae, is diminished (Figure 2). Since the mutant precursor molecules do not accumulate in time but disappear, we have to presume that they are subjected to degradation.

A similar kind of defect was observed in two other cases of adult GSDII (Hermans

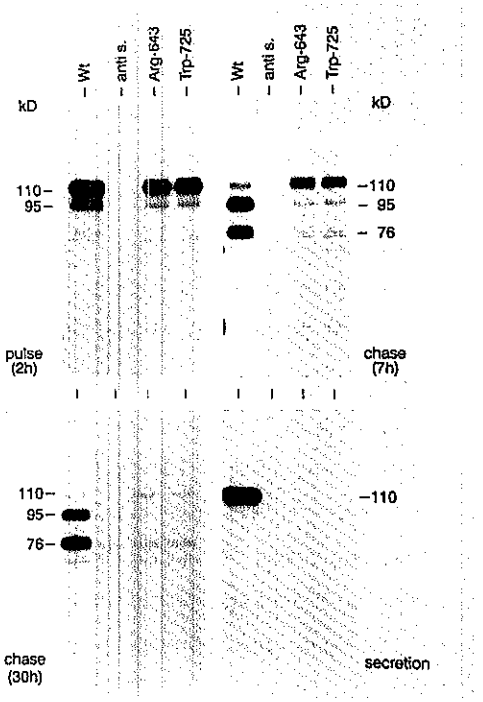

Figure 1: Synthesis and processing of wild type and mutant lysosomal α-glucosidase species in transfected COS cells.
COS cells transfected with wild type and mutant cDNA constructs were labelled with Tran-[^{35}S]-methionine and harvested directly (pulse) or after a subsequent period of either 7 or 30 h (chase). Lysosomal α-glucosidase was immunoprecipitated from the cell homogenates and analyzed by SDS-PAGE. To study secretion the enzyme was immunoprecipitated from the culture medium collected after 7 h of chase. The molecular mass (kD) of the wild type and mutant lysosomal α-glucosidase species is indicated.

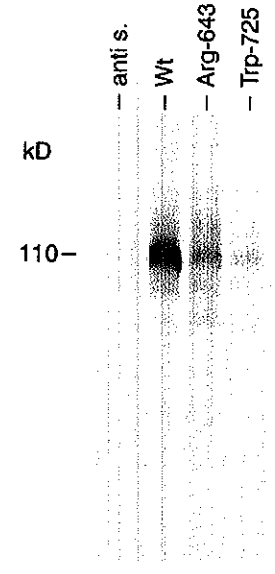

Figure 2: Phosphorylation of wild type and mutant lysosomal α-glucosidase in COS cells.
COS cells were labelled for 4 h with [^{32}P]-phosphate and harvested directly. Lysosomal α-glucosidase was immunoprecipitated from the cell homogenates and analyzed by SDS-PAGE. The molecular mass of the wild type lysosomal α-glucosidase precursor is indicated.

et al., 1993; Hermans, unpublished results), in which the residual lysosomal α-glucosidase activity was unusually low in the patients fibroblasts. Suggestive evidence for a higher residual activity in muscle tissue was obtained in one of these cases (Willemsen et al., 1993). A difference in the expression of the mutant enzyme in muscles compared to fibroblasts could explain also the clinical phenotype of the patient described in this paper.

In conclusion, we have demonstrated that this patient with an adult form of GSDII is a genetic compound. The Gly-643 → Arg and Arg-725 → Trp mutations impair the transport and maturation of lysosomal α-glucosidase in a similar way and they result both in a severe enzyme deficiency. Arg-725 is conserved in the homologous human and rabbit enzymes sucrase and isomaltase, in glucoamylase from *Schwanniomyces occidentalis* and in α-glucosidase from *Candida tsukubaensis* pointing to an essential role (Hunziker et al., 1986; Dohmen et al., 1990; Kinsella et al., 1991; Chantret et al., 1992). With respect to the effect of the Gly-643 → Arg substitution it is notable that defective transport was also found in another case of adult GSDII in which an Asp to Glu substitution had occurred at the nearby position 645 (Hermans et al., 1993). It remains to be clarified how these mutations exert their effect since they are not predicted to change the secondary structure significantly (Chou and Fasman 1978).

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CHAPTER 9

IDENTIFICATION OF A POINT MUTATION IN THE HUMAN LYSOSOMAL α-GLUCOSIDASE GENE CAUSING INFANTILE GLYCOGENOSIS TYPE II.

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IDENTIFICATION OF A POINT MUTATION IN THE HUMAN LYSOSOMAL α-GLUCOSIDASE GENE CAUSING INFANTILE GLYCOGENOSIS TYPE II

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Summary. Two patients in a consanguineous Indian family with infantile glycogenosis type II were found to have a G to A transition in exon 11 of the human lysosomal α-glucosidase gene. Both patients were homozygous and both parents were heterozygous for the mutant allele. The mutation causes a Glu to Lys substitution at amino acid position 521, just three amino acids downstream from the catalytic site at Asp-518. The mutation was introduced in wild type lysosomal α-glucosidase cDNA and the mutant construct was expressed *in vitro* and *in vivo*. The Glu to Lys substitution is proven to account for the abnormal physical properties of the patients lysosomal α-glucosidase precursor and to prevent the formation of catalytically active enzyme. In homozygous form it leads to the severe infantile phenotype of glycogenosis type II.

Lysosomal α-glucosidase (EC 3.2.1.3) deficiency leads to glycogenosis type II (GSDII), a disease inherited as an autosomal recessive trait (1). The clinical phenotype of GSDII varies. Severe infantile and mild, late onset, forms of disease occur (2). Heterogeneity exists also at the molecular level. Partial or complete deficiencies of lysosomal α-glucosidase mRNA have been reported (3-6), and studies on the expression of lysosomal α-glucosidase in fibroblasts and muscle cells of patients have indicated the existence of allelic diversity (7-15). The human lysosomal α-glucosidase gene is in addition polymorphic (16-27).

In this paper we report on the identification of a mutant allele which we discovered previously in a consanguineous Indian family from South Africa (14, Family C). Two siblings were born with the severe infantile form of GSDII. Lysosomal α-glucosidase activity was undetectable in cultured fibroblasts of the patients, and processed forms of

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lysosomal α-glucosidase were found to be fully deficient. The lysosomal α-glucosidase precursor seemed to be about 5 kD smaller than the normal 110 kD wild type precursor, as judged from the mobility on SDS-PAGE. This apparent difference in size was demonstrated to reside in the protein backbone rather than in the carbohydrate side chains. Both patients were homozygous whereas both parents were heterozygous for the size abnormality (14).

The molecular analysis, described below, reveals the point mutation in the lysosomal α-glucosidase gene that causes the physical abnormality of the mutant enzyme precursor, and the lack of proteolytic processing and catalytic activity. It explains the severe clinical phenotype of the patients.

MATERIALS AND METHODS

DNA amplification. Genomic DNA was extracted from cultured fibroblasts by following standard procedures. 0.5 μg DNA was PCR amplified using sense and antisense primers complementary to intron sequences of lysosomal α-glucosidase (unpublished results). These primers were designed to amplify all coding sequences. PCR was performed according to the method described by Sambrook et al. (28). To generate dsDNA fragments, 100 pmol of each primer was used. ssDNA was obtained by asymmetric PCR using 0.5 pmol of one and 50 pmol of the other primer and the dsDNA fragments as starting material. DNA fragments were amplified in 30 cycles using optimal denaturation, annealing and extension temperatures. DNA fragments were sequenced after a Centricon-100 purification step (Amicon) using the T7 polymerase sequencing kit (Pharmacia LKB Biotechnology Inc.) and appropriate primers.
Construction of mutants. Site-directed mutagenesis using the method of Kunkel (29) was carried out as described before (30). The following oligonucleotides, synthesized on an Applied Biosystems 381A DNA synthesizer, were used: Lys-521 TGACATGAACAAGCCTTCCAA; Asp-521 ACATGAACGACCCTTCCAACT and Gln-521 TGACATGAACCAGCCTTCCAA.
Transient expression in COS cells. Wild type and mutant cDNA constructs were cloned in the eukaryotic expression vector pSG5 (31) and transfected to COS cells as described (32). Lysosomal α-glucosidase activity in the cell homogenates was measured with 4-methylumbelliferyl-α-D-glucopyranoside (4-MU) and with glycogen as described (30). The COS cells were labeled with [^{3}H]-leucine (Amersham U.K.) at 65 h after transfection and lysosomal α-glucosidase was immunoprecipitated 4 h later (pulse) or after 4 h labeling followed by 17 h chase, and analyzed by SDS-PAGE as described (9).
***In vitro* transcription/translation.** Wild type and mutant cDNA constructs in pSG5 were linearized and used as templates in a transcription reaction. *In vitro* transcribed RNA was used for *in vitro* translation as described (32).
Immunocytochemistry. The procedure described by van Dongen et al. (33) was used for the immunocytochemical localization of lysosomal α-glucosidase in transiently transfected COS cells.

RESULTS AND DISCUSSION

To identify the mutation causing infantile GSDII in this consanguineous Indian family it was decided to PCR-amplify all the exonic regions of the lysosomal α-

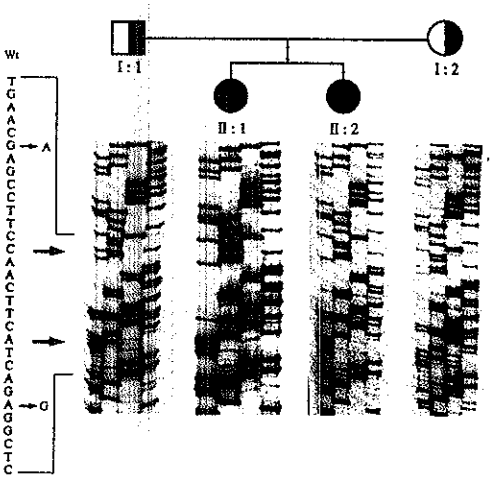

Fig. 1. Partial DNA sequence of exon 11 of the lysosomal α-glucosidase gene from members of a consanguineous Indian family with GSDII.
The order of the lanes is G, A, T, C. The position of the G to A and A to G transitions are indicated with fat arrows. Wt is the wild type sequence which corresponds to the nucleotide sequence 1775 to 1805 in ref. 19 and with the sequence 1556 to 1586 in ref.22.

glucosidase gene (19) of one of the two affected siblings and to determine the sequence.

We had sequenced about 20% of the coding regions when two mutations in exon 11 were found (Fig. 1). The first concerns a transition of G to A and the second a transition of A to G. The patient was homozygous for both mutations, as can be expected in a consanguineous family. Subsequent analysis of the corresponding genomic region of the other affected sibling and the parents revealed the same mutations. But, both parents were heterozygous for the mutations, whereas the second affected sibling was homozygous like her sister (Fig. 1).

The A to G transition is a known DNA polymorphism and does not give rise to an amino acid alteration at position Arg-527 (19,22). However, the G to A transition results in an amino acid change. The negatively charged amino acid residue Glu-521 is replaced by Lys having a positive charge. The mutation is situated only three amino acids downstream from the catalytic site at Asp-518 (30).

It was not immediately evident how a single amino acid substitution in the middle of the primary structure could lead to the formation of a precursor with a seemingly lower than normal molecular mass (14). But, a similar effect had been observed when

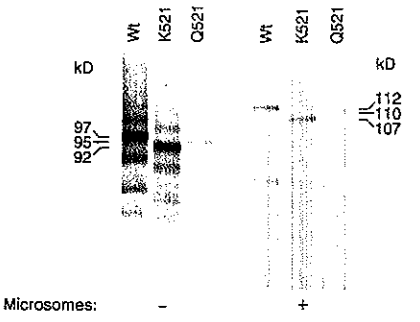

Fig. 2. *In vitro* translation of lysosomal α-glucosidase.
Wild type and mutant cDNA's were transcribed *in vitro* and the mRNA's were translated in a rabbit reticulocyte lysate in the presence or absence of canine pancreatic microsomes. The proteins were labeled with [^{35}S]-methionine. Lysosomal α-glucosidase was immunoprecipitated and analyzed by SDS-PAGE. Wt stands for wild type (Glu-521). The amino acid residues are indicated in the one-letter code: K is Lys and Q is Gln.

the catalytic site residue Asp-518 was substituted by Asn or Gly (30). Also then, the removal of a single negative charge had resulted in an increased electrophoretic mobility of the precursor of lysosomal α-glucosidase upon SDS-PAGE. Thus, we reasoned the substitution of Glu-521 by Lys to be responsible for the physical and functional abnormalities of lysosomal α-glucosidase in this particular case of GSDII.

The first experiment to sustain our assumption was to introduce the Glu-521 to Lys mutation in the wild type cDNA and to perform *in vitro* transcription and translation. In addition, Glu-521 was altered to Gln. The results are illustrated in Fig. 2. The Lys-521 *in vitro* translation product runs approximately 5 kD ahead of the Glu-521 wild type precursor, both in unglycosylated (without microsomes) and glycosylated form (with microsomes). This mimics the results that were obtained when the synthesis of the lysosomal α-glucosidase precusor in the patients fibroblasts was studied with or without inhibition of glycosylation by tunicamycin (14). The Gln-521 mutant precursor, with a less drastic alteration of charge, has an intermediate mobility. Thus, the single amino acid substitution of Glu-521 by Lys accounts, indeed, for the 5 kD shift in electrophoretic mobility.

To confirm the effect of the Glu-521 to Lys mutation on the biosynthesis, the proteolytic processing and the function of lysosomal α-glucosidase, the wild type and mutant cDNA constructs were transiently expressed in COS cells. In this experiment, one additional mutation was introduced to evaluate the influence of the charge of the amino acid residue at position 521. It concerns the substitution of Glu-521 by Asp whereby the

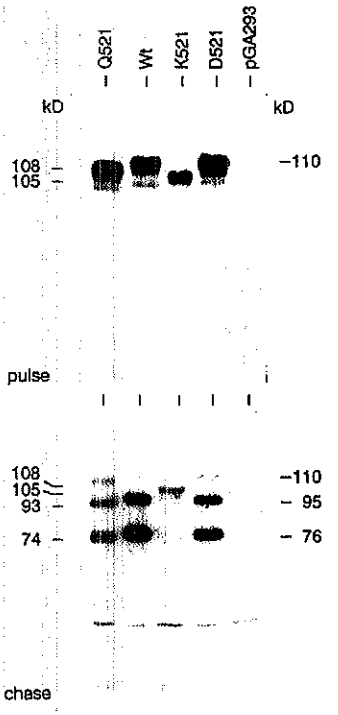

Fig. 3. Synthesis and processing of wild type and mutant lysosomal α-glucosidase species in transiently transfected COS cells.
COS cells transfected with wild type and mutant cDNA constructs were labeled for 4 h with [^{3}H]-leucine and harvested directly (pulse) or after a subsequent period of 17 h (chase). Lysosomal α-glucosidase was immunoprecipitated and analyzed by SDS-PAGE. The relative molecular mass of wild type and mutant lysosomal α-glucosidase species is indicated. Wt stands for wild type (Glu-521). The amino acid residues are indicated in the one-letter code as in Fig. 2, D is Asp. pGA293 is the E. coli ß-Gal construct.

negative charge is maintained. The biosynthesis of lysosomal α-glucosidase was assayed at 65 h after transfection. The results are illustrated in Fig. 3. In a 4 h pulse period the usual 110 kD precursor (32) is synthesized by COS cells transfected with the wild type cDNA construct. COS cells transfected with the mutant Lys-521 cDNA produce a 105 kD precursor. Thus, the apparent size reduction is the same as in the patients fibroblasts (14). The mutant Gln-521 precursor has an intermediate mobility as in the *in vitro* translation system. The substitution of Glu-521 by Asp has no effect on the electrophoretic behaviour. The wild type precursor is converted within 17 h of chase to a 95 kD intermediate and a 76 kD mature species. The mutant Lys-521 precursor is not

Table 1. Catalytic activities of lysosomal α-glucosidase mutants in transiently transfected COS cells measured 90 h after transfection

Type of mutation	Catalytic activity	
	cells*	cells**
Wild type	567.9	2901.0
Glu-521 → Lys	31.2	295.4
Glu-521 → Gln	40.4	333.3
Glu-521 → Asp	68.0	497.5
ß-Gal construct	38.4	358.8

The activity is expressed as * nmol 4-MU/ mg protein/ h or as ** nmol glucose/ mg protein/ h.

processed, but degraded. Also this lack of proteolytic processing reflects fully the molecular defect observed in the patients fibroblasts. The Gln-521 and Asp-521 mutations do not interfere with the maturation process. The altered electrophoretic mobility of Gln-521 is maintained during proteolytic processing.

The lysosomal α-glucosidase activity in the transfected COS cells and in the culture media are given in Table 1. COS cells transfected with E. coli ß-galactosidase cDNA were taken as a reference. No activity above background was measurable after substitution of Glu-521 by Lys. This was as expected since activity was not measurable either in the patients fibroblasts (14). Also substitution of Glu-521 by Gln resulted in a complete loss of activity. It is surprising that even the homologous alteration of Glu to Asp deprives lysosomal α-glucosidase for more than 90% of its activity. This may be related to the close proximity of Glu-521 to the catalytic site at Asp-518 (30).

Finally we investigated the intracellular localization of the Lys-521 mutant protein in transfected COS cells using immunocytochemistry. COS cells expressing the Lys-521 cDNA showed a diffuse network of labeled structures extending from the nuclear envelop instead of the normal lysosomal labeling pattern (data not shown). This indicates that the mutant precursor is not transported to the lysosomes.

In conclusion, we have demonstrated that the Glu-521 to Lys substitution in exon 11 of the lysosomal α-glucosidase gene prevents the formation of catalytically active enzyme. Patients homozygous for this mutation are expected to have a severe infantile form of glycogenosis type II.

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CHAPTER 10

NEW MISSENSE MUTATIONS (GLY-478 → ARG AND MET-519 → THR) IN LYSOSOMAL α-GLUCOSIDASE RESPONSIBLE FOR INFANTILE GLYCOGEN STORAGE DISEASE TYPE II IN TWO DUTCH PATIENTS.

NEW MISSENSE MUTATIONS (GLY-478 → ARG AND MET-519 → THR) IN LYSOSOMAL α-GLUCOSIDASE INTERFERE WITH TRANSPORT, DECREASE STABILITY AND RESULT IN SUGAR CHAIN MODIFICATION

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Summary. Inherited deficiency of lysosomal α-glucosidase (acid maltase) results in glycogen storage disease type II. This paper reports on the identification of the mutant alleles in two patients both with an infantile form of the disease. Genetic analysis revealed that in these patients only one allele was transcribed into mRNA and that this allele harboured in each case a distinct mutation. In one case the mutant allele was characterized by a G-1432 to A transition in exon 9 of the lysosomal α-glucosidase gene resulting in the substitution of Gly-478 by Arg. In the other case the mutant allele contained a T-1556 to C transition in exon 11 causing the substitution of Met-519 by Thr. Interestingly, Met-519 is adjacent to the catalytic site residue Asp-518.
Each of the mutations was introduced in the wild type lysosomal α-glucosidase cDNA and expressed in COS cells to analyze its effect. The synthesis of the mutant precursors was not disturbed and their phosphorylation was normal, but the formation of mature lysosomal enzyme was impaired in both cases. As a consequence a much lower lysosomal α-glucosidase activity was expressed in COS cells transfected with the mutant than the wild type cDNA's. The mutant precursors were secreted at 9.4% and 16.5% of the normal level and were subjected to aberrant sugar chain modifications. These results suggest that both the Gly-478 → Arg and the Met-519 → Thr mutations delay the transport of lysosomal α-glucosidase through the Golgi complex.

INTRODUCTION

It is the primary function of lysosomal α-glucosidase (EC 3.2.1.3) to degrade glycogen which has entered the lysosomes by way of autophagy. Inherited deficiency of lysosomal α-glucosidase causes glycogen storage disease type II (GSDII, Pompe's disease)(McKusick, 1990). The accumulation of glycogen provokes functional failure of heart and skeletal muscle. The disease is clinically and biochemically heterogeneous. Some severely affected patients were described with a complete deficiency of lysosomal

α-glucosidase synthesis. Other patients were reported to have a normal synthesis of the 110 kD precursor but were impaired in their processing to more mature lysosomal α-glucosidase species with molecular masses of 95 kD, 76 kD and 70 kD. Mutant lysosomal α-glucosidase species with abnormal physical properties were found to be rare (Reuser *et al.*, 1978, 1985, 1987; Beratis *et al.*, 1978, 1983; Reuser and Kroos, 1982; Martiniuk *et al.*, 1986, 1990a; Hoefsloot *et al.*, 1988, 1990a; Van der Ploeg *et al.*, 1989).

The distinct defects in the realization of lysosomal α-glucosidase activity have obviously to do with genetic heterogeneity, and evidence for this view is emerging. Several different mutations have been identified among patients with GSDII (Hermans *et al.*, 1991a, 1993; unpublished results; Zhong *et al.*, 1991). So far only one point mutation has been found more than once among unrelated patients (Hermans *et al.*, unpublished results).

In this study we have analyzed the lysosomal α-glucosidase genotype of two patients, both with an infantile form of GSDII (460LAD and 75RD100). In cultured fibroblasts of these patients the residual lysosomal α-glucosidase activity was about 4% for glycogen and less than 1% for the artificial substrate 4-methylumbelliferyl α-D-glucopyranoside. The synthesis of the 110 kD precursor was found to be normal, but the posttranslational processing to be impaired. Only trace amounts of 95 kD and 76 kD processed forms of the enzyme species were detectable on immunoblots of cell lines 460LAD and 75RD100. Some enzyme was present in the lysosomes (Reuser *et al.*, 1987).

The genetic analysis described in this paper demonstrates that both patients are genetic compounds and that one of their mutant alleles is not transcribed into mRNA. The second allele of both patients had in each case a different point mutation leading to a different amino acid substitution. The mutations were introduced in wild type lysosomal α-glucosidase cDNA by site directed mutagenesis and their precise effect on the biosynthesis of lysosomal α-glucosidase was studied.

MATERIAL AND METHODS

DNA amplification and sequencing. DNA and RNA were extracted from cultured fibroblasts following standard procedures (Sambrook *et al.*, 1989). cDNA synthesis was

carried out using Amersham's cDNA Synthesis System Plus and oligo (dT) primers. PCR amplification using intronic or exonic primers specific for lysosomal α-glucosidase was as described (Hermans *et al.*, 1993).

Slot-blot analysis and allele specific oligonucleotide hybridization. Slot-blot analysis and allele specific oligonucleotide hybridization (ASO) were performed exactly as described before (Hermans *et al.*, 1993).

Site directed mutagenesis. Site directed mutagenesis was carried out as described before (Hermans *et al.*, 1991b). The following oligonucleotides, synthesized on an Applied Biosystems 381A DNA synthesizer were used: Arg-478 GCCGCTGATTAGGAA GGTATG and Thr-519 TGGATTGACACGAACGAGCCT.

Transient expression in COS cells. Wild type and mutant cDNAs were cloned in the eukaryotic expression vector pSG5 (Green *et al.*, 1988). The transfection of COS cells was carried out as described before (Hoefsloot *et al.*, 1990b). The activity of lysosomal α-glucosidase in the cell homogenates and the culture media was measured with 4-methylumbelliferyl-α-D-glucopyranoside (4-MU) and with glycogen as described (Hermans *et al.*, 1991b).

Labelling conditions with Tran-[^{35}S]-methionine (ICN), or carrier-free [^{32}P]-phosphate (Amersham) were as described (Reuser *et al.*, 1985). Cells were pulse-labelled for 2 or 4 h and harvested either directly or after a subsequent chase of 7 or 30 h. Lysosomal α-glucosidase was immunoprecipitated from the cell extracts and the culture media and analyzed by SDS-PAGE as described (Hasilik and Neufeld, 1980a, 1980b; Reuser *et al.*, 1985). Some samples were deglycosylated with endoglycosidase H, endoglycosidase F or N-glycanase according to the instructions of the manufacturer (Boehringer/ Sanbio).

***In vitro* transcription/translation.** Wild type and mutant cDNA constructs in pSG5 were linearized with Bgl II and used as templates in a transcription reaction. *In vitro* transcribed RNA was used for *in vitro* translation as described (Hoefsloot *et al.*, 1990a).

Immunocytochemistry. Immunocytochemistry on transiently transfected COS cells was performed exactly as described before (Hoefsloot et al. 1990b).

RESULTS

Sequence analysis

Genomic DNA was isolated from cultured fibroblasts of two unrelated patients both with a severe infantile form of GSDII, and the exons of the lysosomal α-glucosidase gene (GAA) were PCR-amplified to determine the molecular lesions. Most PCR products were sequenced directly, but some were cloned prior to sequencing. In the latter case four separate clones were analyzed.

In patient 460LAD two transitions were found in the coding region of the lysosomal α-glucosidase gene when compared with the wild type sequence published by Hoefsloot *et al.* (EMBL entry number Y00839): G-1432 to A, and C-2808 to T. Only the

G to A transition resulted in an amino acid alteration, being a substitution of Glu-478 by Arg. The silent C to T transition represented a new polymorphism (Table 1). The patient was heterozygous for both these transitions, since the normal lysosomal α-glucosidase sequence occurred along with the mutant sequence.

Also in patient 75RD100 only one mutation was found in the coding region that resulted in an amino acid change. The T-1556 to C transition was causing the substitution of Met-519 by Thr. Interestingly, this mutation was located in the active site of lysosomal α-glucosidase, only one amino acid downstream from the catalytic site at Asp-518 (Hermans *et al.*, 1991b). A second base pair change found in 75RD100 was a silent C-642 to T transition and was described before as a polymorphism (Table 1) (Martiniuk *et al.*, 1990). Patient 75RD100 was heterozygous for both these transitions. Thus, the sequence analysis uncovered in each patient only one mutant allele.

Since the patients presented with a severe infantile form of GSDII we investigated whether perhaps the alleles with the wild type sequence were not expressed. To this end RNA was extracted from cultured fibroblasts of the patients and reverse-transcribed into cDNA. Fragments spanning the mutations were then PCR-amplified and analyzed on slot-blots for the occurrence of the wild type and mutant sequences by allele-specific oligonucleotide hybridization. The cDNA fragments of both patients were found to hybridize only with the mutant and not with the wild type oligonucleotides whereas the corresponding cDNA fragments from a healthy individual hybridized only with the wild

Table 1: Base pair substitutions (Comparison with the lysosomal α-glucosidase cDNA sequence in the EMBL/Genbank (Y00839).

nucleotide position*	exon	cDNA	patient	amino acid alteration	reference
460LAD					
1432	9	G	A	Gly-478 → Arg	
2808	20	C	T	none	
75RD100					
642	3	C	T	none	1
1556	11	T	C	Met-519 → Thr	

* Nucleotide position counted from the initiation codon of the precursor. (1) Martiniuk *et al.*, 1990b.

type oligonucleotides (Figure 1). These results demonstrated that in both patients only the lysosomal α-glucosidase allele with the defined mutation was expressed, and that this allele resulted in a Gly-478 → Arg substitution in patient 460LAD and in a Met-519 → Thr substitution in patient 75RD100.

Synthesis of mutant lysosomal α-glucosidase

To study the effect of the Gly-478 → Arg and the Met-519 → Thr substitutions on the biosynthesis and function of lysosomal α-glucosidase, the corresponding mutations were introduced in the wild type cDNA sequence by site-directed mutagenesis and expressed in COS cells. Two independent clones of each construct were tested. The enzymatic activities of lysosomal α-glucosidase measured in the COS cells at 90 h after transfection are given in Table 2. An antisense cDNA construct served as a negative control. Transfection with the wild type cDNA resulted in a significant increase of activity for both the artificial 4-MU substrate and the natural substrate glycogen. Both mutated enzymes were expressed at about 5% of the wild type activity level irrespective

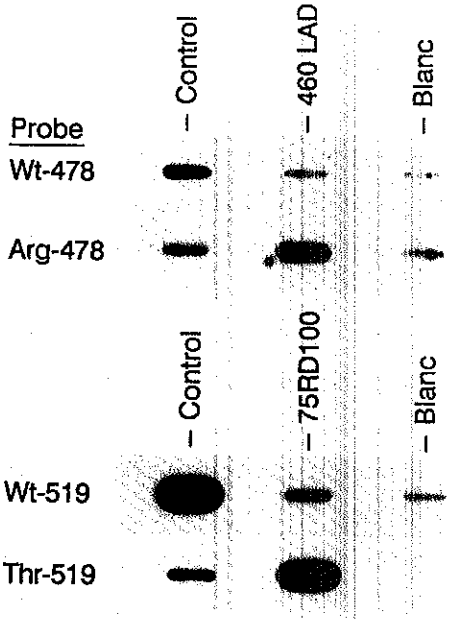

Figure 1: Allele specific oligonucleotide hybridization of PCR-amplified cDNA.
PCR products were denatured and applied to Hybond N^+ membranes using a slot-blot apparatus and probed with [^{32}P]-labelled oligonucleotides for the presence of either normal (Wt) or mutant cDNA sequences.

Table 2: Catalytic activities of lysosomal α-glucosidase mutants in transiently transfected COS cells 96 h after transfection.

Type of mutation	Catalytic activity		
	Cells[a]	Medium[a]	Cells[b]
COS cells	42.3 ± 3.6[c]	7.1 ± 0.4	115 ± 15
Wild type	663.6 ± 32.6	276.7 ± 10.9	4118 ± 201
Gly-478 → Arg	75.9 ± 0.4	5.4 ± 0.1	278 ± 10
Met-519 → Thr	87.8 ± 4.1	5.2 ± 0.2	265 ± 16

The activity is expressed as [a] nmol 4-MU/ mg protein/ h or as [b] nmol glucose/ mg protein/ h. [c] Indicated is average ± SEM of 6 assays.

of the substrate used. No activity above background was measured in the culture medium of COS cells transfected with the mutant cDNA constructs whereas lysosomal α-glucosidase was secreted at approximately 40 times the background level by COS cells transfected with wild type lysosomal α-glucosidase cDNA.

The synthesis and proteolytic processing of the wild type and the mutant Arg-478 or Thr-519 lysosomal α-glucosidase species were assayed by pulse-chase labelling at 48 h after transfection (Figure 2). In COS cells transfected with either the wild type cDNA or the mutant cDNA's a normal 110 kD lysosomal α-glucosidase precursor was synthesized in a 2 h pulse period with Tran-[^{35}S]-methionine. Within 7 h of chase, practically all wild type precursor molecules of 110 kD were converted to 95 kD and 76 kD species. In contrast, only minute amounts of the 95 kD and 76 kD species had been formed from the mutant precursors. The mutant precursor proteins were still present although they were severely reduced in amount after 7 h of chase. After 30 h of chase the molecular pattern of the wild type species was almost the same as after 7 h of chase. However, the mutant enzymes species had disappeared almost completely. Secretion of the mutant precursors could be demonstrated but was less than normal (16.5% for the Arg-476 and 9.4% for the Thr-519 precursor), and the apparent molecular mass of the secreted mutant precursor species was slightly higher than of the wild type species.

Labelling with [^{32}P]-phosphate was performed to determine whether the mannose 6-phosphate recognition marker was formed properly. The degree of phosphorylation of

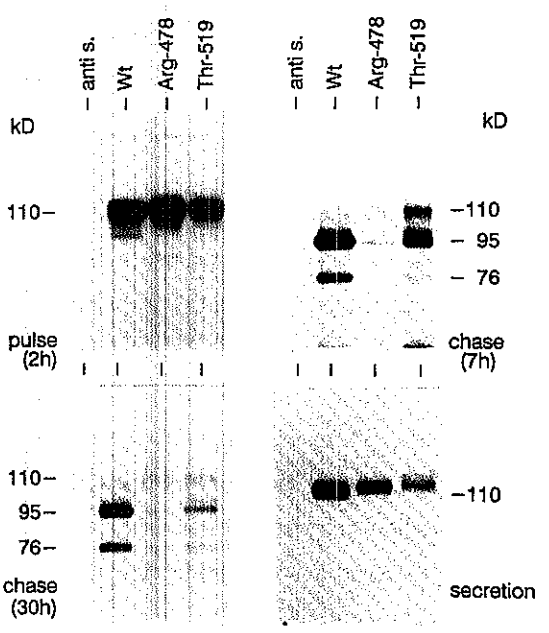

Figure 2: Synthesis and processing of wild type and mutant lysosomal α-glucosidase species in transfected COS cells.
COS cells transfected with wild type and mutant cDNA constructs were labelled with Tran-[^{35}S]-methionine and harvested directly (pulse) or after a subsequent period of either 7 or 30 h (chase). Lysosomal α-glucosidase was immunoprecipitated from the cell homogenates and analyzed by SDS-PAGE. To study secretion the enzyme was immunoprecipitated from the culture medium. The molecular mass (kD) of the wild type lysosomal α-glucosidase species is indicated.

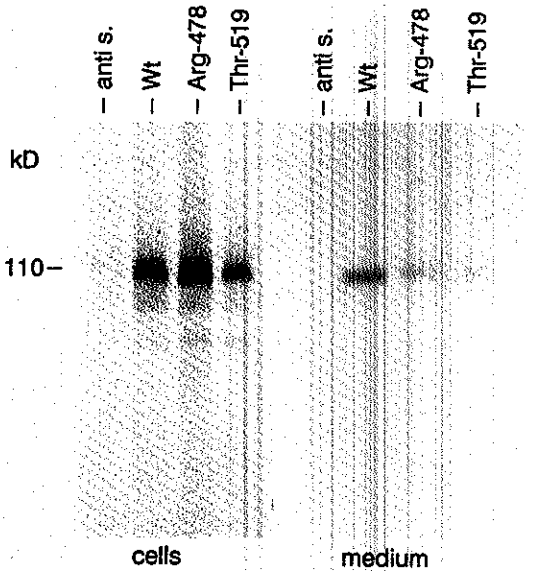

Figure 3: Phosphorylation of wild type and mutant lysosomal α-glucosidase in COS cells.
COS cells were labelled for 4 h with [^{32}P]-phosphate and harvested directly. Lysosomal α-glucosidase was immunoprecipitated from the cell homogenates and the culture medium and analyzed by SDS-PAGE. The molecular mass of the wild type lysosomal α-glucosidase precursor is indicated.

the intracellular wild type and mutant enzyme species was the same (Figure 3, cells), but there was much less phosphorylated mutant than wild type precursor secreted in the medium (Figure 3, medium).

Glycosylation of mutant lysosomal α-glucosidase

The intracellular Arg-478 and Thr-519 precursor species had the same apparent molecular mass as the wild type species. However, the mutant precursor molecules that were secreted into the culture medium appeared to have a higher molecular mass than the secreted wild type precursor. It is known that the intracellular precursor of lysosomal α-glucosidase contains exclusively high mannose type of oligosaccharide chains whereas the secreted precursor has obtained several complex type of chains. The switch from high mannose to complex glycosylation may result in an increase of the apparent molecular mass (Wisselaar *et al.*, 1993). Hence it was considered that the mutant precursors were subjected to aberrant sugar chain modification. To investigate this possibility the secreted precursors were digested with endoglycosidase H (Endo H), endoglycosidase F (Endo F) or N-glycanase (Glyco N). Figure 4 shows that Endo H treatment did reduce the molecular mass but that it did not eliminate the difference between the wild type and mutant species. Complete deglycosylation with endo F and Glyco N was not obtained but there was a clear additional mass reduction compared to Endo H treatment. More important, the molecular mass difference between the Arg-476 and the wild type precursor disappeared and the difference between the Thr-519 and the wild type precursor became extremely small. This indicates that aberrant glycosylation is responsible for the decreased electrophoretic mobility of the secreted mutant precursors. The primary amino acid changes seemed to contribute hardly. This was confirmed by SDS-PAGE of the unglycosylated wild type and mutant precursor proteins produced in an *in vitro* transcription and translation system (Figure 5). The apparent molecular mass of all three naked proteins was essentially the same.

DISCUSSION

The two severely affected patients, analyzed in this study, were found by sequence

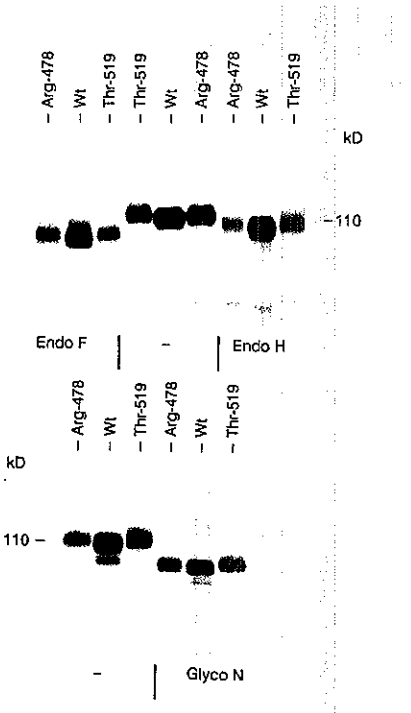

Figure 4: Deglycosylation of secreted wild type and mutant lysosomal α-glucosidase species. Transfected COS cells were labelled with Tran-[^{35}S]-methionine as described in the legend of Figure 2. The enzyme immunoprecipitated from the culture medium was treated with Endo H, Endo F or Glyco N. The molecular mass of the wild type lysosomal α-glucosidase precursor is indicated.

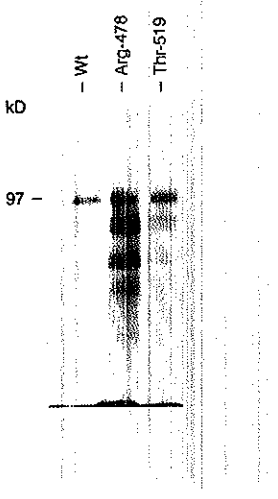

Figure 5: *In vitro* translation of lysosomal α-glucosidase. Wild type and mutant cDNA's were transcribed *in vitro* and the mRNA's were translated in a rabbit reticulocyte lysate. The proteins were labeled with [^{35}S]-methionine. Lysosomal α-glucosidase was immunoprecipitated and analyzed by SDS-PAGE. The molecular mass of the wild type unglycosylated lysosomal α-glucosidase precursor is indicated.

analysis to have each only one mutation in the coding region of the lysosomal α-glucosidase gene that could be responsible for the disease. For patient 460LAD it concerned the G-1432 to A transition in exon 9 resulting in the substitution of Glu-478 by Arg. The mutation in cell line 75RD100 was the T-1556 to C transition causing the substitution of Met-519 by Thr. The patients were heterozygous, which implies that they had one allele with the identified mutation and a second allele without mutations in the coding region. The promoter region of the gene and the complete intron sequences were not analyzed for the possible occurrence of mutations, but we did study whether both alleles of each patient were transcribed. The PCR fragments obtained after reverse-transcription of mRNA were proven by allele specific oligonucleotide hybridization to represent only the alleles with the defined mutations. It was therefore concluded that the patients were genetic compounds. Since the patients were suffering from a severe infantile form of GSDII it was anticipated that the Gly-478 → Arg and the Met-519 → Thr substitutions would have a drastic effect on enzyme function.

This was confirmed by the expression of the mutant lysosomal α-glucosidase species in COS cells. Neither of the two amino acid substitutions had an obvious effect on the formation of the 110 kD precursor. Also the incorporation of phosphate was normal demonstrating that the mannose 6-phosphate recognition marker was formed correctly. Thus, transport of the mutant precursor from the endoplasmic reticulum to the Golgi complex was not disturbed. However, there was less than normal secretion of the precursors and less than normal maturation to 95 kD and 76 kD species. This suggests that a defect in the biosynthesis of lysosomal α-glucosidase occurs before segregation of the lysosomal and secretory pathways; this is before exit of the Trans Golgi Reticulum. The nature of this defect could be delayed transport. This explains why only little mature enzyme is formed and only a low residual lysosomal α-glucosidase activity is realized in the COS cells.

It was not purely coincidental that the Gly-478 → Arg and the Met-519 → Thr mutations were found to have a similar effect. The cell lines 460LAD and 75RD100 were studied in combination since a great similarity had been observed in the clinical presentation of the patients and the molecular nature of the lysosomal α-glucosidase deficiency (Reuser *et al.*, 1987). What went unnoticed in these earlier studies on cultured fibroblasts was the apparently increased molecular mass of the secreted precursors. The

removal of high-mannose type of sugar chains by Endo H treatment did not eliminate this abnormality but the difference between wild type and mutant precursors virtually disappeared by Endo F and Glyco N treatment. We therefore conclude that the apparent molecular mass difference is caused by altered carbohydrate processing resulting in an increase of the number of sialic acid residues.

It is notable that no molecular mass difference exists between the intracellular mutant and wild type precursors, neither in the patients fibroblasts (Reuser *et al.*, 1987), nor in the transfected COS cells (Figure 2). Also the 95 kD and 76 kD processed forms of the mutant enzyme had a normal molecular mass. Based on these findings it is our hypothesis that delayed transport through the Golgi complex favours complex glycosylation the same way as when exit of glycoproteins from the Golgi is prevented by Brefeldin A. This complex glycosylation will result in the addition of more sialic acid residues than usual and this may aversely affect binding to the mannose 6-phosphate receptor. As a consequence there may be selective transport of normally glycosylated precursor molecules to the lysosomes, while the highly sialylated molecules are preferentially secreted.

Although it is impossible to correlate the observed abnormalities in the biosynthesis of lysosomal α-glucosidase directly to the Met-519 → Thr substitution there are a number of interesting findings. The Met-519 → Thr substitution found in patient 75RD100 is located immediately adjacent to Asp-518, the essential carboxylate in the catalytic site of lysosomal α-glucosidase. Studies on the role of potentially important amino acid residues in the catalytic site region have shown that the primary structure is very critical for proper enzyme function (Hermans *et al.*, 1991b). Also the Met-519 → Thr substitution affects the catalytic activity. While the amount of secreted molecules is reduced to 9.4%, the catalytic activity is abolished completely. Furthermore, the Met-519 → Thr substitution interferes with intracellular transport in analogy to other natural and artificially introduced mutations in the catalytic site region. The latter is likely to be caused by a change in secondary structure of the protein since the substitution eliminates an α-helix (Chou and Fasman, 1978).

The Gly-478 → Arg substitution of patient 460LAD is present in a region of lysosomal α-glucosidase of which the specific function is not yet known. However, the region containing Gly-478 is highly conserved in enzymes that show homology with

lysosomal α-glucosidase like rabbit and human isomaltase, *Schwanniomyces occidentalis* glucoamylase and *Candida tsukubaensis* α-glucosidase (Hunziker *et al.*, 1986; Dohmen *et al.*, 1990; Kinsella *et al.*, 1991; Chantret *et al.*, 1992). Apparently, conservation of this region is of functional importance. The Chou & Fasman algorithms predict the creation of an extra β-pleated sheet by the Gly-478 → Arg substitution (Chou and Fasman, 1978).

In conclusion, we have demonstrated that both the Gly-478 → Arg and the Met-519 → Thr substitutions prevent the formation of sufficient amounts of catalytically active enzyme. In combination with a null allele, both these mutations lead to the severe infantile form of GSDII.

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CHAPTER 11

GENERAL CONSIDERATIONS

GENERAL CONSIDERATIONS

A large number of mutations have been identified in lysosomal enzymes over the past few years (for review see Hoefsloot, 1991; Neufeld, 1991). A major part of the experimental work described in this thesis focuses on the identification and characterization of mutant alleles of the lysosomal α-glucosidase gene. Furthermore the aim of the experimental work was to provide more insight in the structure-function relationships of lysosomal α-glucosidase. The results presented in this thesis provide definite proof that GSDII is genetically heterogeneous as anticipated from earlier studies on the biosynthesis of lysosomal α-glucosidase in clinical variants. Consistent with the fact that GSDII is a rare genetic disorder, the majority of the mutations discovered so far is unique. All but two patients are genetic compounds. Even patients previously classified on a clinical and biochemical basis as belonging to the same group (Reuser *et al.*, 1987) appeared to have distinct mutant alleles (see *Chapter 2,* Table 2).

Types of mutations and their effect

Mutation events are the driving force of evolution. They provide for biological variation and are subjected to selective forces resulting in species which fit best into their natural environment. In a clinical context, however, it is more natural to regard mutations as processes that threaten the integrity of the (human) organism. If deviations from the common nucleotide sequence are found, the significance of these variations has to be assessed.

The majority of mutations are of little consequence to the organism since they occur in parts of the genome which lack coding capacity. When aberrations are present within regions of the genome that code for a protein, they may have functional consequences and can lead to a state of disease.

Each mutation has its own specific effect on the function of the protein in question and has informative value for predicting the clinical phenotype. In Table 1 an attempt has been made to predict the clinical consequences of different types of mutations in the genes coding for lysosomal enzymes. Using Table 1 it is important to realize that the lysosomal

Table 1: Effects and consequences of different kind of mutations on lysosomal enzymes.

domain	mutation	Primary effect	Consequence	Clinical outcome
gene	complete deletion		no mRNA/ no protein	severely affected
promoter	deletion insertion point mutation	none	none	healthy
		reduced rate of transcription	reduced amount of mRNA and protein	H→S[1,2]
		no transcription	no mRNA and no protein	severely affected
		introduction of a new initiation codon	aberrantly sized mRNA and protein	severely affected[3]
3' untranslated region	deletion insertion point mutation	none	none	healthy
		loss of polyadenylation site	none	healthy
			reduced amount of mRNA and protein	H→S[2]
			normal amount of mRNA, no protein	severely affected
			no mRNA and no protein	severely affected
intron	deletion insertion point mutation	none	none	healthy
		exon skipping and defective splicing	stable, aberrantly sized mRNA, but (partial) loss/gain of coding sequences combined with frame shift	H→S[4]
			stable, aberrantly sized mRNA, but (partial) loss/gain of coding sequences combined with in-frame change of number of amino acids	H→S[4]
			labile, aberrantly sized mRNA, but (partial) loss/gain of coding sequences combined with frame shift	H→S[4]
			labile, aberrantly sized mRNA, but (partial) loss/gain of coding sequences combined with in-frame change of number of amino acids	H→S[4]
			no mRNA and no protein	severely affected

domain	mutation	Primary effect	Consequence	Clinical outcome
exon	deletion insertion	aberrantly sized mRNA	(partial) loss/gain of coding sequences combined with in-frame change of number of amino acids	H→S[4]
			(partial) loss/gain of coding sequences combined with frame shift	H→S[4]
		unstable mRNA	reduced amount of aberrantly sized protein	H→S
		no mRNA	no protein	severely affected
	point mutation	none	none	healthy
		introduction or deletion splice site	(partial) loss/gain of coding sequences combined with frame shift	H→S[4]
			(partial) loss/gain of coding sequences combined with in-frame change of number of amino acids	H→S[4]
		amino acid alteration	none	healthy
			reduced enzyme activity unstable protein abnormal processing and /or transport of precursor loss/gain of a glycosylation site	H→S[2]
			loss of initiation codon loss of stopcodon	H→S
			introduction premature stopcodon	H→S[4]

[1] H→S, ranging from healthy to severely affected. [2] Severity is dependent on the critical threshold of the enzyme involved. [3] Results in loss of signal sequence. [4] Severity is dependent on the position of the mutation in the coding region of the gene.

storage diseases, with the exception of Hunter disease and Fabry disease, are autosomal recessive disorders and that two mutant alleles contribute equally to the clinical phenotype.

Table 1 shows that a direct prediction of the clinical phenotype can only be made when the gene is completely deleted. In all other cases the effect of the mutations needs to be investigated on the mRNA or protein level. Some illustrative examples of mutations and their effects are discussed in the next paragraphs.

Complete gene deletions have been reported in X-linked Hunter disease (mucopolysaccharidosis type II, α-L-iduronate sulphate sulphatase). As expected, the two patients concerned were severely affected (Flomen *et al.* 1992; Beck *et al.* 1992). In autosomal recessive lysosomal storage diseases complete gene deletions are disastrous only if the patient is a homozygote. Such homozygous patients have not yet been identified. Small mutations in the promoter regions of lysosomal genes have not been reported either but a large deletion, including the promoter region, was found in the hexosaminidase-α gene (Myerowitz and Hogikyan, 1987). A French Canadian patient with the classical (severe) form of Tay-Sachs disease had a 7.6 kb deletion in both alleles which included part of intron 1, all of exon 1 and extended 2 kb upstream past the putative promoter region of the gene. mRNA and protein were undetectable.

An interesting example of a mutation in the 3' untranslated region (3'UTR) of a lysosomal gene was found in aspartylglucosaminuria, a disease enriched in the genetically isolated Finnish population. A severely affected patient was shown to be homozygous for a 876 base pair deletion in the 3'UTR of the aspartylglucosaminidase mRNA, which leaves the coding region intact. Despite the lack of all three putative polyadenylation sites, the transcript is polyadenylated. Alternative sites upstream of the deletion breakpoint are probably used. A normal amount of aberrantly sized mRNA was synthesized but enzyme could not be detected in the patients fibroblasts. Interestingly, an enzymatically active enzyme could be synthesized in an *in vitro* translation system (Ikonen *et al.*, 1992).

When a point mutation in a splice site results in a complete deficiency of mRNA it will lead to the most severe clinical phenotype. Such a mutation was for instance found in Tay-Sachs disease. It concerns a G → C transition in the first nucleotide of intron 12 of the hexosaminidase-α gene (Arpaia *et al.*, 1988; Ohno and Suzuki, 1988).

An insertion or deletion does not always result in an enzyme deficiency. The sphingolipid activator protein (SAP-1) presents a nice example. Several healthy individuals have a 9 base pair insertion between nucleotides 777 and 778 of the

prosaposin precursor (Nakano *et al.*, 1989). The in-frame change of the amino acid sequence does apparently not interfere with the activator function of SAP-1. On the other hand when the 9 base pair insertion extends to the 5' site into a 33 base pair insertion it leads to partial loss of function. A metachromatic leukodystrophy (MLD) patient homozygous for this 33 base pair insertion had an intermediate form of MLD and died at the age of 22 (Zhang *et al.*, 1990).

A deletion or insertion which does give rise to a frame shift leads mostly to a premature stop of protein synthesis. *Chapter 7* shows that a single base pair deletion in exon 2 of the lysosomal α-glucosidase gene does not allow the formation of lysosomal α-glucosidase. The effect of a frameshift or a premature stopcodon on enzyme function is position dependent. Hunter disease is illustrative. Two patients were described with a point mutation introducing a premature stopcodon. A patient with a stopcodon at amino acid position 172 of the 550 amino acid residues long sequence of α-L-iduronate sulphate sulphatase had a severe form of Hunter disease whereas a patient with a premature stop at position 443 was diagnosed with a milder intermediate form of the disease (Flomen *et al.*, 1992; Sukegawa *et al.*, 1992).

Mutations introducing or deleting glycosylation sites have in general an unpredictable effect. For instance deletion of the seventh glycosylation site of lysosomal α-glucosidase does not affect enzyme function (*Chapters 4 and 5*) and occurs as a polymorphism among American blacks (*Chapter 6*), whereas deletion of the second glycosylation site is deleterious (*Chapter 4*). In arylsulfatase A the loss of one of the three glycosylation sites does not have any effect on the enzyme's function, whereas loss of the only glycosylation site of SAP-1 results in rapid degradation of the enzyme (Gieselmann *et al.*, 1989; Kretz *et al.*, 1990; Rafi *et al.*, 1990).

Genotype-phenotype correlation

The identification of characteristic mutations is to some extent predictive for the clinical phenotype. In the Ashkenazi Jewish population, Niemann-Pick disease types A and B (acid sphingomyelinase deficiency) occur in a relatively high frequency. Type A is a severe neurodegenerative disease while type B is characterized by a later age of onset and little or no neurological involvement. Two mutations were identified which point to a

genotype-phenotype correlation. An Arg-496 → Leu substitution in homozygous form is associated with the A subtype whereas patients presenting with the nonneurological B subtype are either homozygous for an Arg-608 deletion (ΔArg-608) or are heterozygous (Arg-496 → Leu/ ΔArg-608) (Levran *et al.*, 1991a; 1991b).

A genotype-phenotype correlation also has been proposed in MLD. A point mutation destroying a splice donor site was shown to prevent the formation of arylsulfatase A mRNA, and a Pro-426 → Leu substitution to result in the formation of an active but unstable enzyme (Polten *et al.*, 1991). Gieselmann *et al.* (1991) screened a number of patients for the occurrence of these alleles and found that patients homozygous for the splice site mutation suffered from the late infantile form of MLD. Homozygotes for the Pro-426 → Leu allele manifested either the juvenile or the adult phenotype. Heterozygotes have the intermediate form of MLD.

Also in GSDII an example of a genotype/phenotype correlation was found (*Chapter 7*). A juvenile patient had two different lysosomal α-glucosidase alleles, one with a single base pair deletion (ΔT-525) which was functionally silent and the other with a Pro-545 → Leu substitution capable to produce a low level of catalytically active enzyme. An adult patient was found to be homozygous for the Pro-545 → Leu allele. These results suggest that gene dosage accounts for the different clinical phenotypes of these two patients with GSDII.

A problem with a seemingly straightforward prediction of the clinical phenotype is found in Gaucher disease (glucocerebrosidase deficiency) in which three clinically different subtypes (I, II, and III) can be distinguished according to age of onset and neurological involvement. The most common mutation in Gaucher disease among the Ashkenazi Jews is an Asn-370 → Ser substitution. It accounts for ~ 75% of disease producing alleles in this population (Beutler, 1992). Patients homozygous for this allele always have the relatively mild type I phenotype. There is, however, a large variability within this group of patients with respect to the age of onset of this disease. The average onset is at 27 years but some individuals can have serious problems at a much earlier age while others remain free of symptoms. Variability is seen even between siblings which makes the prediction of the clinical phenotype of the homozygous Asn-370 → Ser mutation impossible.

The above examples illustrate that there is a clear genotype-phenotype correlation

in some of the lysosomal storage diseases but that such correlation lacks in others. The lack of correlation could be due to the variation in genetic background and epigenetic factors which contribute to the ultimate effect of the mutant gene.

These secondary factors are expected to be most influential when the residual activity of the mutant enzyme comes close to the critical threshold. This critical threshold was defined by Conzelmann and Sandhoff (1983/1984) as the level of residual activity just high enough to degrade all the substrate that enters the lysosomes. In Tay-Sachs disease the critical threshold for β-hexosaminidase activity was estimated to be around 10 - 15% of the average control value (Leinekugel *et al.*, 1992). For lysosomal α-glucosidase the critical threshold is estimated to be around 25% (Reuser *et al.*, 1987).

Taking the clinical diversity among Asn-370 → Ser homozygotes in Gaucher disease it can be assumed that the residual activity of the Ser-370 form of glucocerebrosidase is close to the critical threshold. When the level of residual activity reaches the critical threshold the capacity of the defective lysosomal enzyme to prevent the accumulation of substrate is going to depend on the influx rate of substrate into the lysosomes. This rate is determined by the large number of genes, directly or indirectly controlling the overall metabolic status of the cell and a complex of cellular (transport) processes. For the lysosomal system these processes include specifically lipid flow, autophagy and heterophagy.

It are these parameters controlled by the genetic background that contribute to the ultimate clinical phenotype. The contribution of the genetic background in the development of the clinical phenotype is emphasized by the cell-type specific expression of lysosomal pathology.

Finally, the clinical course of a disease may be influenced by epigenetic factors such as diet and exercise which can modify the metabolic status of the cell and the organism as a whole.

In conclusion, mutation analysis *per se* is mostly not sufficient for prediction of the clinical phenotype of a patient unless the *in vivo* effect of a mutant allele or combination of alleles is known from family studies and the residual performance of the mutant protein is significantly below or above the critical threshold. However, mutation analysis can reveal important information on the structure-function relationship of lysosomal enzymes and proteins in general.

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SUMMARY

SAMENVATTING

SUMMARY

Lysosomal α-glucosidase is capable of degrading glycogen completely to glucose by hydrolysing both the α-1,4 and α-1,6 linkages. Inherited deficiency of lysosomal α-glucosidase results in the lysosomal accumulation of glycogen, a condition known as glycogen storage disease type II (GSDII, Pompe's disease). Different clinical forms of this disease have been reported varying in time of onset, cardiac and liver involvement, severity of muscle weakness and life expectation. Genetic heterogeneity has been predicted by the variety of abnormalities observed in the biosynthesis of lysosomal α-glucosidase in fibroblasts and muscle cells of the patients. This thesis deals with the structure-function relationship of lysosomal α-glucosidase and the identification of mutations in GSDII.

Chapter 1 gives an overview of the α-glucanases to which class of enzymes lysosomal α-glucosidase belongs. The general reaction mechanism of the α-glucanases is described and the structural and functional homologies of the different α-glucanases are discussed.

Chapter 2 starts with an introduction about the synthesis, the intracellular transport and the proteolytic processing of lysosomal enzymes in general and of lysosomal α-glucosidase in particular. The role of the enzymes involved in the glycogen metabolism and the related storage diseases is outlined. Special attention is paid to glycogen storage disease type II. The second part of *Chapter 2* refers to the experimental work described in *Chapters 3 to 10*. It focuses on the functional analysis of the catalytic site domain of lysosomal α-glucosidase, and the glycosylation sites, and further on the analysis of mutations in the lysosomal α-glucosidase gene that cause glycogen storage disease type II.

Chapter 3 reports on the characterization of the catalytic site of lysosomal α-glucosidase. The catalytic base was labelled using the active site-directed inhibitor conduritol B epoxide and was identified as Asp-518. The functional properties of several amino acid residues in the catalytic site domain were studied in more detail.

In *Chapter 4* it is demonstrated that all seven potential glycosylation sites of lysosomal α-glucosidase are used, and that two sites are located in a C-terminal peptide that is cleaved off during maturation. It is shown that only the loss of the second glycosylation site at Asn-233 interferes with the transport and maturation of lysosomal α-

glucosidase.

The *Chapters 5 to 10* deal with the molecular characterization of the mutant alleles of patients with GSDII. An American black patient with adult GSDII is described with two different mutant alleles. One is silent and the other codes for three amino acid substitutions. The Asp-645 → Glu substitution is proven by expression studies to be responsible for the deleterious effect on enzyme function (*Chapter 5*). The two other amino acid substitutions, Val-816 → Ile and Thr-927 → Ile (deleting the seventh glycosylation site of lysosomal α-glucosidase) are shown to be common polymorphisms among American blacks (*Chapter 6*).

Two other cases of adult and one case of juvenile GSDII are discussed in the *Chapters 7 and 8*. In *Chapter 7* it concerns two unrelated Dutch patients, one with a juvenile and the other with an adult form of GSDII. The patients were found to be homozygous (adult) and heterozygous (juvenile) for a Pro-545 → Leu substitution. The second allele of the juvenile patient was functionally silent, since a one base pair deletion at position 525 resulted in a premature stop of protein synthesis. The Pro-545 → Leu substitution does not lead to a complete loss of function. Expression in COS cells shows that 8% of the normal capacity of lysosomal α-glucosidase is maintained. Since the adult patient has two copies of this allele compared to the juvenile patient only one, these data are an excellent example of a genotype-phenotype correlation in GSDII.

The molecular genotype of a third adult patient is reported in *Chapter 8*. The patient is a genetic compound, with a Gly-643 → Arg substitution encoded by one mutant allele and an Arg-725 → Trp by the other. Both mutations have a similar effect when expressed in COS cells, they impair the transport and maturation of lysosomal α-glucosidase and are thereby responsible for the severe enzyme deficiency of the patient.

The molecular genotypes of three cases of infantile GSDII are described in *Chapters 9 and 10*. Two novel alleles with a mutation in the catalytic site domain of lysosomal α-glucosidase were found. The first concerned the substitution of Glu-512 → Lys (homozygous) in two siblings of a consanguineous Indian family from South Africa. The other mutation was a Met-519 → Thr substitution. The patient carrying this mutant allele was a genetic compound with a second silent allele. The third patient with infantile GSDII was also a genetic compound with a silent allele and an allele coding for Arg-478 in stead of Gly. When these mutant alleles were expressed in COS cells, all three amino

acid substitutions appeared to have a drastic effect on the maturation and function of lysosomal α-glucosidase in accordance with the severe clinical phenotypes of the patients.

The general considerations in *Chapter 11* are focused on the type of mutations in lysosomal genes and their possible effect and on the value of mutation analysis for predicting the clinical phenotype.

The studies described in this thesis have expanded the knowledge about the structure-function relationship of lysosomal α-glucosidase and have given a first real view of the genetic heterogeneity in GSDII.

SAMENVATTING

Het lysosomale enzym α-glucosidase is in staat glycogeen volledig af te breken tot glucose door zowel de α-1,4 als de α-1,6 verbindingen te hydrolyseren. Een erfelijke deficiëntie van dit enzym leidt tot een lysosomale stapeling van glycogeen, een aandoening die bekend staat onder de naam glycogeen stapelingsziekte type II of te wel de ziekte van Pompe (in dit proefschrift wordt de afkorting GSDII gebruikt). Er zijn meerdere klinische vormen van deze ziekte bekend, die verschillen wat betreft de leeftijd waarop de symptomen zich manifesteren, het optreden van hart en lever pathologie, de ernst van de spierzwakte en de levensverwachting van de patiënt. Genetische heterogeniteit binnen deze ziekte werd vermoed op grond van de diverse abnormaliteiten die waargenomen werden m.b.t. de synthese van het lysosomale enzym α-glucosidase in huid en spiercellen van patiënten. In dit proefschrift wordt de relatie tussen structuur en functie van het lysosomale enzym α-glucosidase bestudeerd en worden mutaties beschreven die aanleiding geven tot GSDII.

Hoofdstuk 1 geeft een overzicht van de α-glucanasen, tot welke klasse van enzymen ook het lysosomale α-glucosidase behoort. Het reactiemechanisme dat voor alle α-glucanasen hetzelfde is wordt beschreven en de structurele en functionele overeenkomsten van de α-glucanasen worden besproken.

Hoofdstuk 2 begint met een inleiding over de synthese, het intracellulaire transport en de proteolytische modificaties van lysosomale enzymen in het algemeen, en van het lysosomale α-glucosidase in het bijzonder. De rol van de enzymen betrokken bij het glycogeen metabolisme en de gerelateerde glycogeen stapelingsziekten wordt aangegeven. Aan GSDII wordt speciale aandacht besteed. Het tweede gedeelte van *Hoofdstuk 2* verwijst naar het experimentele werk dat beschreven wordt in de *Hoofdstukken 3 tot en met 10*. De nadruk ligt op de functionele analyse van het katalytische centrum en de glycosyleringsplaatsen van het lysosomale α-glucosidase en op de bestudering van mutaties in het lysosomale α-glucosidase gen die leiden tot GSDII.

De karakterisering van het katalytische centrum van het lysosomale α-glucosidase wordt beschreven in *Hoofdstuk 3*. De katalytische base werd gelabeld met de remmer conduritol B epoxide en kon worden geïdentificeerd als Asp-518. De functionele eigenschappen van een aantal aminozuren in het katalytische centrum werden uitvoerig

bestudeerd.

Hoofdstuk 4 laat zien dat alle zeven potentiële glycosyleringsplaatsen van het lysosomale α-glucosidase gebruikt worden. Twee van deze plaatsen bevinden zich in het C-terminale uiteinde van het enzym, dat tijdens de "maturatie" verloren gaat. Uit de experimenten blijkt dat het transport en de maturatie van het lysosomale α-glucosidase belemmerd wordt als de tweede glycosyleringsplaats op Asn-233 ontbreekt.

In de *Hoofdstukken 5 tot en met 10* worden mutaties beschreven die gevonden werden in het lysosomale α-glucosidase gen van patiënten met GSDII. Twee verschillende mutante allelen werden gevonden bij een Amerikaanse patiënt van het negroïde ras met de adulte vorm van GSDII. Van een allel wordt geen mRNA afgeschreven terwijl mutaties in het andere allel resulteren in drie aminozuur substituties. Het effect van ieder van de substituties werd bestudeerd door expressie in gekweekte apeniercellen (COS cellen). De Asp-645 → Glu substitutie blijkt de lysosomale α-glucosidase deficiëntie te veroorzaken (*Hoofdstuk 5*). Van de twee andere aminozuur substituties, Val-816 → Ile en Thr-927 → Ile (de laatste leidt tot verlies van de zevende glycosyleringsplaats) wordt aangetoond dat ze als polymorfisme voorkomen in de Amerikaanse zwarte populatie (*Hoofdstuk 6*).

In de *Hoofdstukken 7 en 8* worden twee andere adulte patiënten en een juveniele patiënt beschreven. In *Hoofdstuk 7* betreft het twee niet verwante Nederlandse patiënten. De adulte patiënt bleek homozygoot en de juveniele patiënt heterozygoot te zijn voor een Pro-545 → Leu substitutie. In het tweede allel van de juveniele patiënt werd een deletie van één base paar aangetroffen op positie 525, hetgeen een premature stop in eiwit synthese veroorzaakt. Expressie van de Pro-545 → Leu substitutie in COS cellen laat zien dat de enzym functie niet volledig verloren gaat maar dat 8% van de normale capaciteit gehandhaafd blijft. Aangezien de adulte patiënt twee kopieën van dit allel heeft en de juveniele patiënt slechts een, zijn deze resultaten een goed voorbeeld van een genotype-fenotype correlatie in GSDII.

Het moleculaire genotype van een derde adulte patiënt wordt besproken in *Hoofdstuk 8*. De patiënt heeft twee verschillende mutante allelen. De mutatie in het ene lysosomale α-glucosidase allel leidt tot een Gly-643 → Arg substitutie en die in het andere allel tot een Arg-725 → Trp substitutie. Beide mutaties hebben een vergelijkbaar effect als de expressie in COS cellen bestudeerd wordt. Ze bemoeilijken het transport en de maturatie van het lysosomale α-glucosidase en zijn daardoor verantwoordelijk voor de

enzym deficiëntie.

In de *Hoofdstukken 9 en 10* worden de resultaten gepresenteerd van mutatie analyse in drie gevallen van infantiele GSDII. Er werden twee mutaties gevonden in het katalytische centrum van het lysosomale α-glucosidase. De eerste betreft de substitutie van Glu-521 → Lys (homozygoot) bij twee kinderen uit een consanguïen Indiaas gezin wonend in Zuid-Afrika. De andere mutatie is een Met-519 → Thr substitutie. De patiënt waarbij deze mutatie gevonden werd bleek twee verschillende gemuteerde allelen te hebben. Van het tweede allel wordt geen mRNA afgeschreven. De derde patiënt had ook twee verschillende allelen, de een niet resulterend in mRNA en de ander coderend voor een Arg-478 → Gly substitutie. Expressie in COS cellen toonde aan dat alle drie aminozuur substituties een ingrijpend effect hebben op de maturatie en de functie van het lysosomale α-glucosidase conform de ernstige klinische fenotypen van deze patiënten.

Hoofdstuk 11 concentreert zich op de verschillende soorten mutaties in lysosomale genen en hun mogelijke effect, en op de waarde die gehecht kan worden aan mutatie analyse voor de voorspelling van het klinische fenotype.

De studies in dit proefschrift beschreven hebben het inzicht in de structuur-functie relatie van het lysosomale α-glucosidase vergroot. Verder is een goed beeld verkregen van de genetische heterogeniteit in GSDII.

CURRICULUM VITAE

LIST OF PUBLICATIONS

NAWOORD

CURRICULUM VITAE

9 januari 1963		Geboren te Heerlen.
Juni 1981		Eindexamen gymnasium β, Coriovallum College, Heerlen.
September 1981		Aanvang studie Biologie.
Oktober 1984		Kandidaatsexamen Biologie (B1w), Katholieke Universiteit Nijmegen.
Februari 1988		Doctoraalexamen Biologie, Katholieke Universiteit Nijmegen.
	Hoofdvak	Genetica, Prof. Dr. W.H.G. Hennig en Dr. P. Huijser.
	Bijvakken	Algemene Botanie, Prof. Dr. H.F. Linskens en Dr. P. Van der Kroon. Biochemie, Dr. W. W. de Jong en Dr. C.E.M. Voorter. 1^e graads lesbevoegdheid Biologie.
Maart 1988		Aanvang promotie-onderzoek op de afdeling Celbiologie en Genetica bij Prof. Dr. H. Galjaard en Dr. A.J.J. Reuser, Erasmus Universiteit Rotterdam.
December 1989		Cambridge Certificate of Proficiency in English.

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NAWOORD

Dit proefschrift was niet mogelijk geweest zonder de hulp van vele anderen. Op de eerste plaats komen mijn ouders, die mij altijd gestimuleerd en gemotiveerd hebben. Mam en Pap, zonder jullie steun was ik niet zover gekomen.

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Arnold Reuser dank ik voor zijn uitstekende begeleiding. Arnold, jouw kritische instelling ten aanzien van experimentele resultaten en van artikelen hebben de waarde van dit proefschrift enorm verhoogd: Bedankt! Marian Kroos wil ik bedanken voor het vele werk dat ze voor mij gedaan heeft. Lieve Marian, ik kan me geen fijnere collega toewensen! Esther de Graaff heeft in het kader van haar doctoraal stage met zeer veel enthousiasme een "enorme berg sequence-werk" verricht. Esther, jou inzet en vriendschap heb ik zeer gewaardeerd. Ben Oostra stond altijd klaar als er "moleculair biologische" problemen waren. Ben, ik vond het enorm fijn dat je steeds tijd voor mij had. Alvin Chan, Hillebrand Olthuis, Anita van Oosten en Mattie Bout wil ik bedanken voor het werk dat zij voor mij gedaan hebben als stagiaires. De andere "α-glu"-collega's, Heleen Wisselaar, Lies Hoefsloot, Ans van der Ploeg, Marianne Hoogeveen en Rob Willemsen dank ik voor de plezierige samenwerking en hun bijdrage aan dit proefschrift. Tom de Vries Lentsch wil ik bedanken voor uitstekend fotografische werk en de afwerking van de figuren. Sjozèf van Baal dank ik voor de hulp met de computer.

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Monique

Stellingen behorende bij het proefschrift

STRUCTURAL AND FUNCTIONAL ANALYSIS OF LYSOSOMAL α-GLUCOSIDASE IN RELATION TO GLYCOGEN STORAGE DISEASE TYPE II

Rotterdam, 9 juni 1993

Monique M.P. Hermans

I

Op grond van de genotype-fenotype correlatie in glycogeen stapelingsziekte type II kan uitgesloten worden dat de lysosomale α-glucosidase deficiëntie die veroorzaakt wordt door de Pro-545 → Leu substitutie voorkomt bij patiënten met de infantiele vorm van deze ziekte.

Dit proefschrift

II

De bewering van Martiniuk *et al.* dat de Val-816 → Ile substitutie leidt tot een lysosomale α-glucosidase deficiëntie is onjuist.

Martiniuk et al. 1991. DNA Cell Biol. ***10****:681-687*

Dit proefschrift

III

Als de negatieve lading van Asp-91 bepalend is voor het ontstaan van een disulfidebrug op Cys-92 dan kan het ontbreken van deze lading in het GAA2 allel (Asn-91) van het lysosomale α-glucosidase een verklaring zijn voor de verlaagde affiniteit van dit molecule voor glycogeen.

Swallow et al. 1975. Ann. Hum. Genet. ***38****:391-406*

Martiniuk et al. 1990. Am. J. Hum. Genet. ***47****:440-445*

Wisselaar et al. 1993. J. Biol. Chem. ***268****: 2223-2231*

IV

De grote invloed die de lading van één enkel aminozuur kan hebben op de electroforetische mobiliteit van een gereduceerd eiwit in SDS-PAGE benadrukt de relativiteit van het getal dat met dit systeem aan de moleculaire massa wordt toegekend.

Dit proefschrift

V

Omdat *Saccharomyces cerevisiae* geen Golgi mannosidases bevat die nodig zijn voor de juiste structurering van de N-gebonden koolhydraat ketens van glycoproteïnen van zoogdieren kan deze species niet gebruikt worden voor de produktie van voor therapie geschikte glycoproteïnen.

Romanos et al. 1992. Yeast 8: 423-488

VI

Een eenvoudige manier om onbekende puntmutaties in genen op te sporen is nog niet gevonden.

VII

Non-identiteit van een identieke vrouwelijke tweeling kan verklaard worden uit allel specifieke verschillen tussen het paternale en het maternale X-chromosoom, die tot uiting zullen komen wanneer ten gevolge van de random X-chromosoom inactivatie de bijdrage van het paternale en maternale X-chromosoom niet identiek is in beide embryo's.

VIII

Het begrip penetrantie wordt aangewend om de discrepantie tussen genotype en fenotype bij autosomaal dominant overervende ziekten aan te duiden. Mechanistisch gezien kan er ook bij de autosomaal recessieve ziekten sprake zijn van penetrantie *vs.* non-penetrantie.

IX

De stijging van de langjarige gemiddelde temperatuur van de atmosfeer sinds 1880 wordt in het algemeen in verband gebracht met een door de mens veroorzaakt "broeikaseffect". Daarbij wordt voorbij gegaan aan het feit dat de huidige langjarige gemiddelde temperatuur nog steeds lager is dan die in het jaar 1800.

Roth 1991. VGB Kraftwerktechnik 71:865-870

X

Bij het beoordelen van het milieu effect van een produkt dient niet alleen de afbreekbaarheid van het produkt maar ook de produktie methode in beschouwing genomen te worden.

XI

Het gemiddeld aantal telefoonnummers dat door een telefoonbezitter wordt opgezocht in een telefoonboek rechtvaardigt het jaarlijks hernieuwd uitkomen van dit boek niet.

XII

Géén *tijd* betekent meestal géén priori*tijd*!