IMMUNE RESPONSE TO ENZYME REPLACEMENT THERAPY IN MPS I AND GSD II PATIENTS

By

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THESIS SUMMARY

Enzyme replacement therapy (ERT) has been shown to be an effective treatment strategy for a number of lysosomal storage diseases (LSD's) in both animal models and in human clinical trials. Immune response to replacement proteins however has been reported for a number of LSD and can be a potential complication for therapy. Immune responses to ERT are variable and dependent on both the inherent antigenicity of the protein being infused and the individual patient. I have investigated the immune reactivity of two lysosomal glycosidases, α-D-glucosidase and α-L-iduronidase. Functional deficiencies of these lysosomal enzymes are the cause of the LSD Glycogen storage disease type II (GSD II) and mucopolysaccharidosis type I (MPS I) respectively.

In a phase I/II clinical trial of ERT in MPS I patients, an immune response to α-L-iduronidase was observed in 50% of patients. The MPS I patients that initially had an immune response were shown in this thesis, to develop natural immune-tolerance to α-L-iduronidase after a year of ERT. This finding has positive implications for MPS I patients who are undergoing long term ERT. A specific peptide that mapped to the catalytic element of α-L-iduronidase also appeared to be important in the development and maintenance of the immune response to α-L-iduronidase. In defining sites on the replacement protein, to which antibodies react, ways to improve ERT by minimising or eliminating antibody reactivity may be engineered.

It was postulated that the conservation of structural features in the active sites of glycosidases using a retaining catalytic mechanism, might result in common antigenicity. This study demonstrated that despite limited sequence identity between α-L-iduronidase and α-D-glucosidase, conserved micro-structural features and regions of short sequence

identity could make a major contribution to common glycosidase antigenicity. This common antigenicity in related glycosidases may contribute some background immune reactivity, representative of the major antigenic sites on the missing glycosidase and may account for why immune response to ERT has proven to be less of an issue than initially postulated.

A high incidence of antibody production to the rh-α-D-glucosidase replacement protein has been reported in the literature for GSD II patients treated by ERT. Furthermore, relative to other LSD types, large doses of rh-α-D-glucosidase are required for effective ERT in GSD II patients. In this thesis, the α-D-glucosidase catalytic site was identified as one of the main antigenic regions of this lysosomal protein. It was postulated that stabilising the catalytic site of α-D-glucosidase could increase the amount of protein recovered during purification and partially mask antibody reactivity against this region. This study demonstrated that D-glucose stabilised α-D-glucosidase allowing increased expression and improved purification of the recombinant protein from an over-expressing CHO-K1 cell line. This simple strategy could accommodate the high demand of enzyme required for ERT studies. D-Glucose also enhanced the residual α-D-glucosidase protein/activity in adult-onset GSD II patient cells suggesting the development of D-glucose analogues may be beneficial as an enzyme enhancement therapy for adult-onset GSD II patients. Furthermore, D-glucose-treated rh-α-D-glucosidase had reduced antibody reactivity *in vitro* suggesting that it may also be useful as a modifier of the immune response to ERT in GSD II patients. These findings have positive implications for ERT in GSD II patients.

When considering the safety and efficacy of ERT in LSD patients, it is important to know when an immune response develops, and whether it is maintained over the course of

treatment. It is clear that some LSD patients will develop antibodies against ERT and now there is also some evidence to suggest that these could compromise the health of the patient and therapy efficacy. Therefore, characterising these immune responses becomes crucial for the management of patients receiving long term ERT. An increased understanding of antibody development in enzyme treated LSD patients will aid in engineering ways to avert the potential affects of antibody production and will assist in the delivery of a more effective and safe therapy.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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LIST OF ABBREVIATIONS

The following abbreviations were used in addition to those abbreviations commonly accepted

α-MEM	Minimum Essential Medium Alpha Medium
4MU-	4-methylumbelliferyl-
4MU	4-methylumbelliferone
ABTS	2,2 azine-di(3-ethylbenzhiazoline sulphonic acid)
BCA	Bianchoninic acid
BME	Basal Medium Eagle
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
CHO-K1	Chinese hamster ovary
CNS	Central nervous system
DELFIA	Dissociation-enhanced lanthanide fluorescence immune-assay
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Cimethyl sulphoxide
DS	Dermatan sulphate
EET	Enzyme enhancement therapy
ELISA	Enzyme linked immunosorbent assay
ERT	Enzyme replacement therapy
GH A	Glycosyl hydrolase- clan A
GRT	Gene replacement therapy
GSD II	Glycogen storage disease type II
HCl	Hydrochloric acid
HGMD	Human gene mutation database
HS	Heparan sulphate

K_i	Inhibitory constant
K_m	Michaelis menten constant
LAMP	Lysosomal-associated membrane protein
LSD	Lysosomal storage disorder
MPS	Mucopolysaccharidoses
MPS I	Mucopolysaccharidosis type I
MPS II	Mucopolysaccharidosis type II
MPS III	Mucopolysaccharidosis type III
MPS VI	Mucopolysaccharidosis type VI
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
NaH_2PO_4	Sodium dihydrogen orthophosphate
NaOH	Sodium hydroxide
NB-DGJ	N-butyldeoxygalactonojirimycin
NB-DNJ	N-butyldeoxynojirimycin
OD	Optical density units
PBS	Phosphate-buffered saline
Rag	Recombinase-activating gene
RFU	Relative fluorescence units
rh-	Recombinant human-
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDT	Substrate deprivation therapy
Tris	Tris(hydroxymethyl)aminomethan
V_{max}	Maximum velocity of catalysis

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PUBLICATIONS ARISING FROM THIS STUDY

Kakavanos, R., Turner, C.T., Hopwood, J.J., Kakkis, E.D. and Brooks, D.A. (2003) Immune tolerance in mucopolysaccharidosis type I patients. *Lancet.* **361**: 1608-1613.

Brooks, D.A., **Kakavanos, R.**, and Hopwood, J.J. (2003) Significance of immune response to enzyme replacement therapy for patients with a lysosomal storage disorder. *Trends Molec.r Med.* **9**: 450-453.

Kakavanos, R., Lehn, P., Callebaut I., Meikle, P.J., Parkinson-Lawrence, E.J., Hopwood, J.J., Brooks, D.A. (2006) Common antigenicity for two glycosidases. *FEBS Lett.* **580**: 87-92.

Kakavanos, R, Hopwood, J.J., Lang, D., Meikle, P.J., and Brooks, D.A. (2006) Stabilising normal and mutant α-glucosidase. *FEBS Lett.* Submitted Dec. 2005.

CHAPTER 1:

INTRODUCTION

1.1 The lysosome and the endocytic network

The lysosome was first described by De Duve *et al.,* in 1955 (cited in Hirschhorn and Reuser, 2001) as a membrane-bound vacuole containing hydrolytic enzymes that function at low pH. The discovery of the lysosome, together with the observations of Novikoff and colleagues (Novikoff *et al.,* 1973) led to the recognition of a coordinated endocytic network which involves a number of components, including the early endosome situated at the cell periphery, the late endosome that is in close proximity to the nucleus and the lysosome (Figure 1.1). They form a network that is responsible for the trafficking of newly-synthesised and digestion of endocytosed macromolecules. Macromolecules from the extracellular environment can be delivered to lysosomes through the pathways of endocytosis and phagocytosis, or from the cytosol via autophagy (Kornfeld, 1986). Endocytosis involves the uptake of extracellular fluid by in-folding of the plasma membrane, and formation of a vesicle containing the extra cellular material. This can be achieved by receptor-mediated endocytosis, where the intended molecule is bound to a specific cell surface receptor before being internalised (Goldstein *et al.,* 1985). Alternatively, this process can take place in a fluid phase (adsorptive endocytosis) through non-specific binding to the plasma membrane before uptake. These molecules, once internalised, are delivered to endosomes and lysosomes for degradation (Vellodi, 2005).

Phagocytosis is the process by which the cell engulfs particulate matter (>0.5 μm), such as micro-organisms and cellular debris, from the extracellular space and digests it by lysosomal action. This process involves pseudopodium formation around the particle to be internalised and the formation of a phagosome containing the engulfed particle. The phagosome then fuses with a primary lysosome leading to the degradation of the phagosome's content (Beron *et al.*, 1995). Autophagocytosis, on the other hand, is the

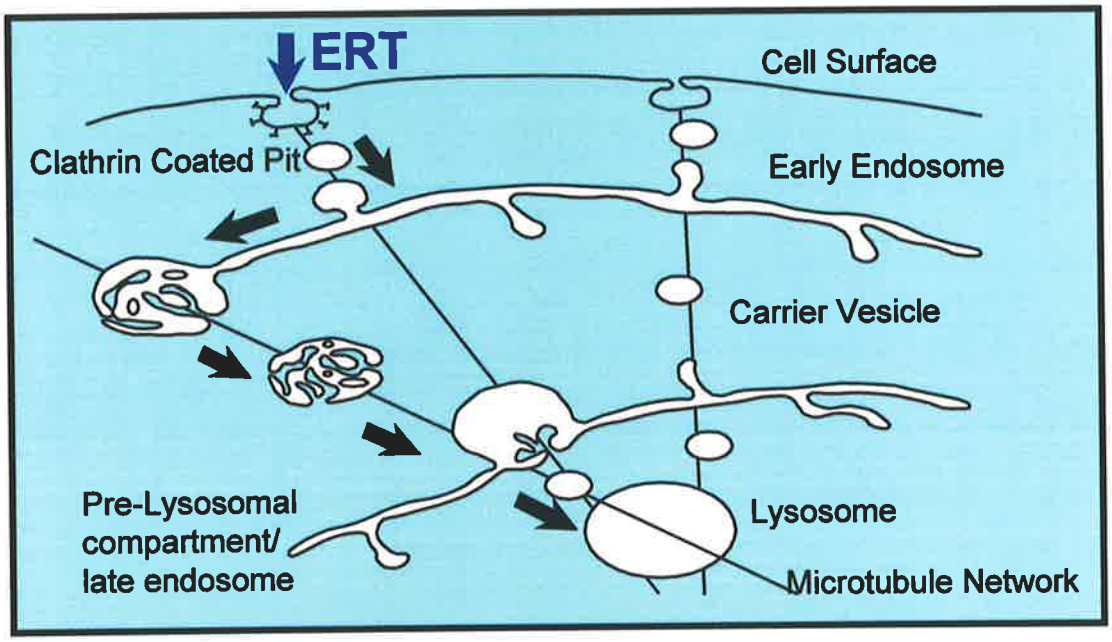


Figure 1.1: Endocytic network and enzyme replacement therapy (ERT)

The endocytic network is a coordinated network that involves a number of components, including the early endosome situated at the cell periphery, endosome carrier vesicles, the late endosome/prelysosomal compartment, that is in close proximity to the nucleus). The components of the endocytic network are arranged on microtubules, which facilitate vesicular trafficking between organelles. During ERT enzyme is internalised from the cell surface by receptor-mediated uptake and trafficks through the different endosome compartments towards its final destination, the lysosome. Adapted from Brooks, 1999.

process by which material within the cell is engulfed (Knowles and Ballard, 1976) and is a process used to recycle cellular components. The organelle is surrounded by a membrane and this structure is termed an 'autophagic vacuole'. The autophagic vacuole fuses with lysosomes and the damaged organelles are degraded by the action of the lysosomal hydrolases.

1.1.1 Synthesis of lysosomal hydrolases and delivery to the lysosome

Lysosomal hydrolases are responsible for the degradation recycling of all types of biological molecules such as proteins, nucleic acid, lipids, glycolipids, glycogen, glycosaminoglycans and the recycling of sulphates and phosphates. Each lysosomal enzyme is involved in a complex pathway that breaks down macromolecules into smaller subunits for either re-use or secretion by the cell. Lysosomal enzymes turn over individually with half-lives that range from one-day to 65-days (Storrie, 1988) and therefore a continuous process of synthesis and delivery of lysosomal hydrolases to the lysosome is required. Lysosomal enzymes are glycoproteins that are synthesised in the endoplasmic reticulum. N-terminal signal sequences aid the translocation of lysosomal enzymes through the endoplasmic reticulum to the lumen where they undergo N-glycosylation and the signal sequence specifically removed (Kornfeld, 1986). Transport to the Golgi apparatus then follows where mannose-6-phosphate residues are added to the lysosomal enzyme by the action of N-acetylglycosamine-1-phosphotransferase (Finean *et al.*, 1984). The acquisition of the mannose-6-phosphate marker is vital for trafficking and targeting to the lysosome, and is the rationale behind some of the therapeutic strategies currently in place for lysosomal storage disorders (LSD), which will be discussed in greater detail in section 1.4. The mannose-6-phosphate residues on lysosomal proteins bind to the mannose-6-phosphate receptors in the trans Golgi apparatus, which consequently

directs their transport to endosomes (von Figura and Hasilik, 1986) where the lower pH causes the receptor-protein complex to dissociate (Gonzalez-Noriega *et al.,* 1980). The enzyme moves into the lysosome and the receptor is recycled to the Golgi apparatus (Duncan and Kornfeld, 1988).

Newly synthesised lysosomal membrane proteins (eg lysosomal-associated membrane proteins (LAMPs)) are sorted through a mannose-6-phosphate-independent pathway. Recognition of a specific cytoplasmic amino acid sequence directs these transmembrane proteins from the *trans*-Golgi network to the lysosome (Janvier and Bonifacino, 2005). A mannose-6-phosphate-independent mechanism for transporting lysosomal proteins to their proper destination has also been proposed for several cell types. This involves oligosaccharide receptors that are found on the plasma membrane, including the asialoglycoprotein and macrophage mannose receptor (Grabowski and Hopkin, 2003). The latter has been targeted for the treatment of the LSD Gaucher disease type 1, because this disease primarily affects the macrophage system to which the mannose receptor is restricted.

1.2 LSD

A number of LSD were first clinically described in the late 19th-century or early 20th-century, prior to the discovery of the lysosome and were usually named after the discovering physician. It was not until 1963 when Hers and colleagues demonstrated the absence of the lysosomal enzyme α-D-glucosidase in Pompe disease (Glycogen storage disease type II, GSD II) that the concept of LSD evolved (Hers, cited in Hirchhorn and Reuser, 2001). As the structure and function of this cellular organelle was defined and

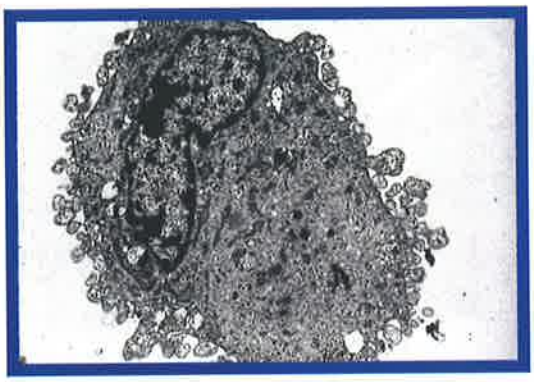
different enzymes were identified, each LSD was linked with a specific lysosomal enzyme(s), and or accumulated substrate(s).

The deficiency of a lysosomal enzyme causes a blockage in the sequential catabolic pathway of substrates within the lysosome, leading to progressive accumulation of intermediate metabolic products (partially degraded or undegraded substrates). As these products accumulate the lysosomes enlarge and occupy more intracellular space, eventually interfering with cellular function (Figure 1.2; Hopwood and Brooks, 1997). As a group, LSD comprise over 40 distinct genetic diseases (Wilcox, 2004). With the technological advances in biochemistry/genetics and increased understanding of lysosomal biology the number of recognised LSD is increasing. Most LSD result from defects of hydrolases; however, defects in enzyme co-activators, membrane transporters, targeting mechanisms for protein localisation to the lysosome and intracellular vesicular trafficking have also been implicated. Although lysosomal proteins are usually ubiquitous, storage occurs only in those cells in which substrate turnover is significant. LSD have been grouped into eight broad sub-categories, based on the type of defect and/or the accumulated substrate as shown in Table 1.1 (Wilcox, 2004). The accumulation of primary storage material, together with a number of secondary biochemical and structural events triggered by the primary storage, is presumed to lead to the pathology associated with LSD.

1.2.1 Prevalence and genetics of LSD

The prevalence of LSD in Australia is one per 7700 live births (Meikle *et al.,* 1999). However, the prevalence of certain LSD can vary greatly depending on ethnicity and geographic origin. For example, Gaucher, Tay Sachs, Niemann-Pick type A diseases and

a.



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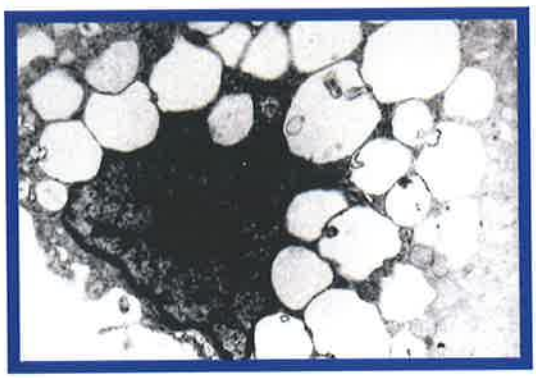

Figure 1.2: Substrate storage in a LSD patient skin fibroblast cell

Electron micrograph of a normal (a) and a LSD patient (b) skin fibroblast cell. Substrate storage can be visualised as electron translucent vacuoles in b.

Table 1.1 Known lysosomal storage disorders

Mucopolysaccharidoses (MPS)
MPS I
MPS II
MPS IIIA, B, C, D
MPS IVA, B
MPS VI
MPS VII
MPS IX
Glycoproteinoses
Aspartylglycosaminuria
Fucosidosis
α-Mannosidosis
β-Mannosidosis
Mucolipidosis I (sialidosis)
Schindler disease
Spingolipidoses
Fabry disease
Farber disease
Gaucher disease (types I-III)
GM1 gangliosidosis
GM2 activator disease
Tay-Sachs disease
Sandhoff diseases
Krabbe disease
Metachromatic leucodystrophy
Niemann-Pick disease (types A and B)
Other lipidoses
Niemann-Pick disease type C
Wolman disease
Neuronal ceroid lipofuscinoses (CLN 1-CLN 8)
Cholesterol ester storage disease
Glycogen storage disease
Glycogen storage disease type II (Pompe disease)
Multiple enzyme deficiency
Multiple sulphatase deficiency
Galactosialidosis
Mucolipidosis II/III and IV
Lysosomal transport and trafficking defects
Cystinosis
Sialic acid storage disease
Danon disease
Chylomicron retention disease with Marinesco-Sjogren syndrome
Chediak-Higashi syndrome
Unknown defects
Geleophysic dysplasia

mucolipidosis IV have a prevalence of more than 50% in the Ashkenazic Jewish population (Mistry, 1999; Natowitcz and Prence, 1996; Schuchman and Miranda, 1997; Edelmann *et al.,* 2002 respectively). The majority of patients with aspartylglucosaminuria are Finnish (Arvio *et al.,* 1993) and mucopolysaccharidosis type III (MPS III) has a high frequency in The Netherlands (Van de Kamp *et al.,* 1981).

LSD are predominantly monogenic disorders and most are inherited in an autosomal recessive manner, with the exception of Fabry disease (X-linked); (Desnick *et al.,* 2001), mucopolysaccharidosis type II (MPS II; X-linked recessive); (Ropers *et al.,* 1977; Wilcox, 2004) and Danon disease (X-linked dominant); (Nishino *et al.,* 2000; Sugie *et al.,* 2002). Genetic heterogeneity is extensive in LSD patients. For some diseases such as Tay Sachs disease, Gaucher disease and Niemann-Pick disease types A and B, many patients have common mutations, while in other diseases, such as Fabry disease, most patients have private mutations. Some mutations appear to correlate with clinical severity or are common in certain ethnic and geographic groups. For example, the Q70X mutation in Hurler syndrome (the rapidly progressing form of mucopolysaccharidosis type I (MPS I)) is more common in Russia and Scandinavia (Voskoboeva *et al.,* 1998). Approximately 50% of all patients with Gaucher disease have one of four common mutations (Koprivica *et al.,* 2000). However, it should be noted that genotype/phenotype correlations are not always accurate, with reports of patients with the same genotype having different clinical outcomes. For example, in Gaucher disease significant differences have been observed in the clinical phenotype between affected siblings sharing the same mutation: one sibling was severely affected while the other had very little manifestation of disease (Beutler and Grabowski, 2001). This suggested that other factors influence clinical presentation and progression and may include the level of residual enzyme activity, treatment and supportive care,

environmental factors and other unknown genetic factors (Conzelmann and Sandhoff, 1983).

1.2.2 Clinical presentation

Although nearly all LSD result from a lysosomal protein deficiency there is great clinical variability, presumably due to the different types of substrate stored and the storage locations. A common feature of these disorders is the progressive nature of the clinical presentation corresponding to the accumulation of substrate over time. LSD are multi-systemic and symptoms usually include a range of somatic and neurological changes. Many LSD share some common characteristics, including short stature, kyphosis, coarse facies, dysostosis multiplex, joint stiffness, heart valve problems, hepatosplenomegaly and corneal clouding (Figure 1.3); (Muenzer, 2004; Wilcox, 2004).

Clinical presentation can vary from the slowly progressing somatic pathology to the more rapidly progressive neurologic pathology. Although some LSD are classified in the literature into clinical subtypes (eg Hurler (rapidly progressing form of MPS I), Scheie (slowly progressing form of MPS I), in reality these subtypes represent the extremes of a continuous clinical spectrum (Beck 2001). The age of onset and severity of symptoms, the organs involved, and effects on the central nervous system (CNS) can vary considerably within a single disorder. Life expectancy varies across the different LSD and depends greatly on the disease severity and the treatment options available. Generally, in the absence of treatment, severely affected LSD patients deteriorate rapidly and die within the first to second decade of life. LSD present a significant emotional, physical and economic burden to the patient but also to the families involved, their community, and the health care system as the need for medical/surgical intervention increases with disease progression.

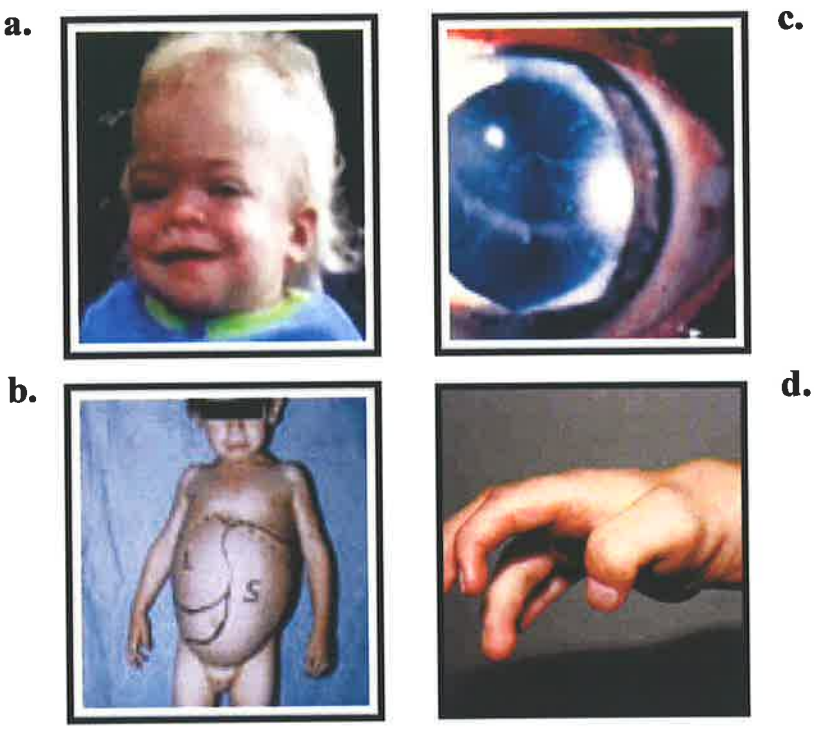

Figure 1.3: Clinical symptoms of LSD

Common clinical symptoms of LSD include coarse facial features (a), hepatosplenomegaly (b), corneal clouding (c) and joint stiffness (d). Photographs obtained with permission from the Genzyzme website: http://www.lysosomallearning.com/healthcare/about/lsd_hc_abt_presentation.asp

This warrants further research, the development of more effective diagnostic tools, and therapeutic strategies to improve the quality of life for these patients.

1.2.3 Diagnosis of LSD

Diagnosis can be challenging, particularly at the slowly progressing end of the clinical spectrum where symptoms can be easily overlooked or misdiagnosed as many of the clinical symptoms characteristic of LSD also appear in other common diseases (Wilcox, 2004). Diagnosis of LSD is usually by clinical presentation but this is often not conclusive and requires validation by laboratory testing. Biochemical diagnosis of LSD involves a variety of assays on blood (white cells and plasma), urine or skin fibroblasts. Assay of specific lysosomal enzyme activity is the primary diagnostic method, but for some extremely rare or recently identified disorders enzymatic assays may not be available. More recently, confirmation of a specific LSD can also be achieved by the mass spectrometric identification and quantification of stored substrates released into blood or urine (Whitfield *et al.,* 2001; Rozaklis *et al.,* 2002; Fuller *et al.,* 2004). In some cases other tests that may suggest the presence of a LSD can be utilised, and include brain magnetic resonance imaging, electroretinogram or biopsy of enlarged tissues for ultrastructural examination (Wilcox, 2004). Prenatal diagnosis of many LSD can be conducted through enzymatic or molecular testing. Foetal fibroblasts circulating in the amniotic fluid can be obtained through amniocentesis and cultured for enzyme assay. Similarly, chorionic villus samples (a small piece of the placenta) can also be analysed for enzyme activity, but this is not frequently utilized, as enzyme levels may be naturally low (Wilcox, 2004). Molecular-based prenatal diagnosis can be done, if the mutations carried by the parents are known. Carrier screening programs for populations at risk can also help lower incidence rates and allow better management of disease.

Diagnosis of an LSD before the onset of irreversible pathology (eg CNS and bone), is important in improving the effectiveness of current and proposed therapies, but also enables the counselling of parents (Meikle and Hopwood, 2003). Currently, diagnosis can take years and families often have more children before the affected child in diagnosed. The development and implementation of presymptomatic diagnosis through newborn screening programs are currently in progress. Initial attempts of newborn screening methods were based on the common feature of lysosomal enlargement and increases in certain lysosomal proteins, such as LAMP-1, (Meikle *et al.,* 1999; Ranierri *et al.,* 1999). However, the inability to delineate between LSD types was a major limitation with these strategies. Recently, the use of electrospray ionization-tandem mass spectrometry has enabled the quantification of specific markers from small amounts of urine or from blood spots to definitively diagnose many of the LSD (Fuller *et al.,* 2004; Meikle *et al.,* 2004; Li *et al.,* 2004; Ramsay *et al.,* 2005). Protein profiling is another screening strategy that is currently being developed, which has the potential to provide increased discrimination between general markers that are elevated in LSD with specific markers that are decreased (Meikle *et al.,* 2005; Meikle *et al.,* 2006 submitted for publication).

In the absence of treatment, once the diagnosis of a LSD is made it is important to effectively manage the disease. Disease management has in the past been generally palliative, involving a multidisciplinary approach and treatment options vary depending on the disorder and the clinical severity. For a number of LSD, disease-specific treatment options that address the underlying enzyme deficiency or the storage problem are currently available and these will be discussed in greater detail in section 1.4.

The focus of this thesis and the remainder of this introduction will be on two LSD, GSD II and MPS I. These two disorders were amongst the first LSD described and respectively result from a functional deficiency of the lysosomal enzymes α-D-glucosidase and α-L-iduronidase. My interest in these two lysosomal enzymes lies in their common catalytic mechanism/catalytic structural element and due to the observation that both have generated immune responses in LSD patients undergoing treatment by enzyme replacement therapy (ERT). Discussions on the clinical phenotype, genetic heterogeneity, protein synthesis and diagnosis for each disorder will follow.

1.3 GSD II

GSD II (also known as Pompe Disease and Acid Maltase Deficiency) is a LSD that results from a defect in the activity of the lysosomal glycosidase α-D-glucosidase (Hers, cited in Hirschhorn and Reuser, 2001). α-D-Glucosidase is responsible for hydrolysing α-1,4- and α-1,6- linkages of oligosaccharides, releasing a glucose molecule (Figure 1.4); (Auricchio *et al.,* 1968; Brown *et al.,* 1970). A deficiency of α-D-glucosidase consequently results in the intralysosomal accumulation of glycogen. α-D-Glucosidase catalyses the complete degradation of glycogen as well as other natural substrates, including maltose, isomaltose and starch (Hirschhorn and Reuser, 2001). Artificial substrates degraded by α-D-glucosidase include 4-methyl-umbelliferyl- (4MU) α-D-glucopyranoside, 6-bromo-2-naphthyl α-D-glucopyranoside and p-nitrophenyl α-D-glucopyranoside and are hydrolysed at rates inversely proportional to their size (Calder and Geddes, 1989).

1.3.1 History of GSD II

In 1932 JC Pompe first described a seven-month old patient who suddenly died from cardiac complications (cardiomegaly); and showed extensive glycogen accumulation

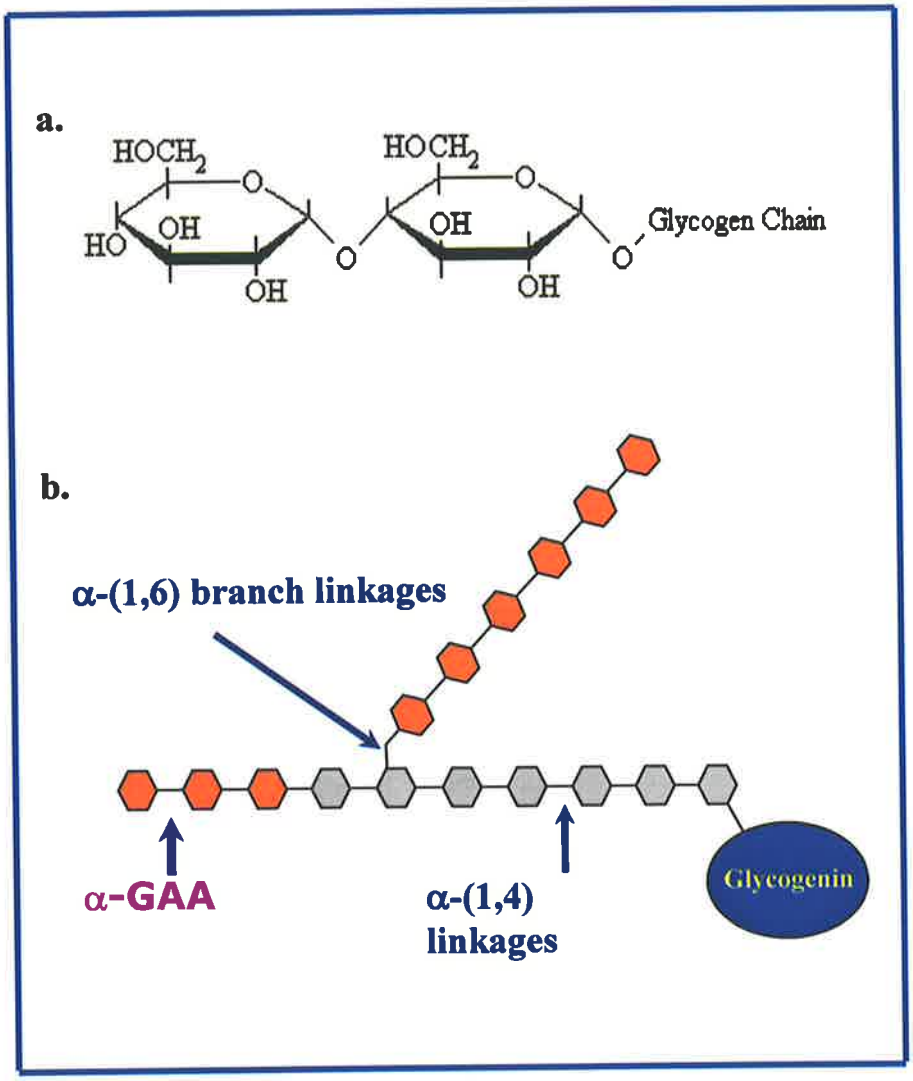

Figure 1.4: Glycogen and α-D-glucosidase activity

The repeating glucose units of glycogen (a) and α-D-glucosidase activity on glycogen (b). Adapted from http://www.med.unibs.it/~marchesi/glycogen.html.

within membrane-bound vesicles (Pompe, cited in Hirschhorn and Reuser, 2001). Pompe also observed that glycogen accumulation was not restricted to the heart but was present in all tissues. The disorder was named Pompe disease after the founding physician. Its metabolic basis was discovered in 1963 after two separate discoveries, the first of which was the delineation of the glycogen metabolism pathway (Cori, cited in Hirschhorn and Reuser, 2001); this led to a classification system of diseases defective in glycogen metabolism and to the re-naming of Pompe disease to GSD II. Although GSD II was one of the most severe forms of glycogen storage disorders, it did not show a defect in the normal glycogen degradation pathway and the storage product, glycogen consisted of normal structure. Around the same period Hers and co-workers made the link between GSD II and the deficiency of a lysosomal enzyme called α-D-glucosidase (Lejeune *et al.*, cited in Hirschhorn and Reuser, 2001). GSD II patients can present with a range of clinical phenotypes that presumably arise from either complete or partial loss of α-D-glucosidase activity. Engel and Dale (1968) first reported an adult-onset form of GSD II in a 46-year-old man, which highlighted the possibility of multiple clinical phenotypes within the one disorder.

1.3.2 GSD II clinical phenotype

Three clinical phenotypes have been reported in the literature: infantile-onset being the most rapidly progressive form; adult-onset being the slowly progressive form; and juvenile-onset, an intermediate of the two extremes. Clinical severity is defined by the age of onset, rate of disease progression and organ involvement (Hirschhorn and Reuser, 2001). GSD II patients present within a spectrum of clinical phenotypes characterised by varying degrees of cardiac and/or skeletal muscle involvement. Although disease severity

correlates with very low residual α-D-glucosidase activity in infantile patients, this distinction is not clearly evident in juvenile- and adult-onset GSD II patients.

1.3.2.1 Infantile-onset GSD II

Infantile-onset GSD II has been segregated into two syndromes (Slonim *et al.*, 2000), classical and 'atypical'. Classical infantile-onset is the most common infantile form of the disorder (Figure 1.5a); infants present within the first months of life with feeding difficulties, poor weight gain, and respiratory problems that can be complicated by pulmonary infection (Figure 1.5a) (Hirschhorn and Reuser, 2001; Van den Hout *et al.*, 2003). Most affected infants have less than 1% α-D-glucosidase activity in skin fibroblasts (Kishnani and Howell, 2004) and clinically present with rapidly progressive muscle weakness and hypotonia leading to what is known as the 'floppy baby' syndrome (Figure. 1.5). Patients also present with enlargement of the heart (cardiomegaly; Figure 1.5b) and, to more variable degrees the tongue (macroglossia; Figure 1.5c) and liver (hepatomegaly) (Hirschhorn and Reuser, 2001; Van den Hout *et al.*, 2003). Motor development is severely delayed, with important milestones such as learning to turn, sit and stand, either not being achieved or lost shortly after acquisition (Hirschhorn and Reuser, 2001; Van den Hout *et al.*, 2003). Death usually occurs in the first year of life primarily due to cardiac and respiratory failure.

Glycogen accumulation is seen in numerous tissues, but predominantly in the liver, heart and skeletal muscle. At autopsy the heart can be as much as three-times its normal size portraying increased ventricular wall thickness and reduction of ventricular cavities. Muscle biopsies have shown the presence of vacuoles staining positive for glycogen as well as the lysosomal enzyme acid phosphatase. When compared to normal, classical

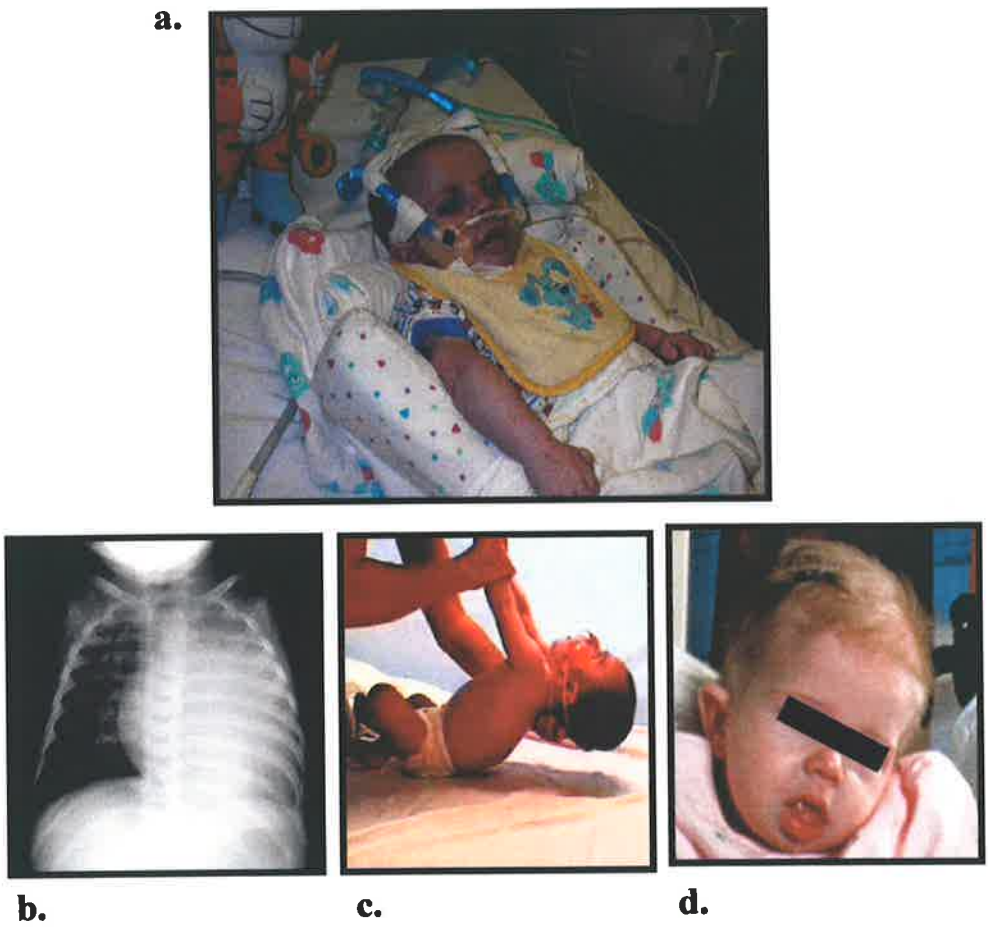

Figure 1.5: Infantile-onset GSD II (Pompe disease)

Clinical manifestations of GSD II : feeding difficulties and respiratory problems (a); cardiomegaly (b); 'floppy baby' appearance (c); and macroglossia (d). Photographs obtained with permisssion from http://www.amda-pompe.org/patients.htm and http://www.lysosomallearning.com/healthcare/about/lsd_hc_abt_pompe.asp.

infantile-onset GSD II patients show up to a 10-fold increase of glycogen in muscle and a three-fold increase in liver (Hirschhorn and Reuser, 2001). Other tissues with marked glycogen accumulation include smooth muscle, endothelial cells, kidney, lymphocytes, the eye, skin and cultured fibroblasts. In the CNS accumulation is more elevated in the spinal cord and brain stem with Schwann cells, spinal neurons, myenteric plexus, astrocytes, oligodendroglia, endothelial cells and pericytes mainly affected.

The 'atypical' infantile-onset form of GSD II is less common and usually diagnosed within the first six-months of life. Cardiomegaly is generally not present and pathology is predominantly evident in the skeletal muscle tissue. Hepatomegaly and macroglossia are also less common; some patients have survived beyond the age of two years, leading to some confusion with the juvenile-onset form of GSD II (Engel *et al.,* 1973).

1.3.2.2 Juvenile-onset GSD II

Clinical signs in juvenile-onset GSD II patients overlap with the extremes of infantile- and adult-onset. Juvenile-onset GSD II patients are usually diagnosed within the first few years of life with progressive muscular symptoms but without cardiac involvement (Hirschhorn and Reuser, 2001; Kishnani and Howell, 2004). Clinical signs begin with delayed motor movements followed by progressive proximal limb muscle and diaphragmatic weakness, leading to pulmonary insufficiency, sleep-disordered breathing and difficulty walking. Death occurs prior to the age of 20 mainly as a consequence of respiratory failure. Cardiomegaly, hepatomegaly and macroglossia are not common clinical manifestations (Hirschhorn and Reuser, 2001).

1.3.2.3 Adult-onset GSD II

Adult-onset GSD II patients are typically asymptomatic in infancy and childhood, with pathology presenting between the second and sixth decade of life (Hirschhorn and Reuser, 2001). During adolescence some adult-onset GSD II patients have been reported to be very athletically active (Swash *et al.,* 1985). The first signs of symptoms include somnolence, morning headache, orthopnea, and exertional dyspnea (Hirschhorn and Reuser, 2001). The predominant symptom is slowly progressive weakness and wasting of axial and proximal limb muscles with a greater tendency for involvement of lower limbs than the upper limbs (Hirschhorn and Reuser, 2001; Kishnani and Howell, 2004). Muscles show variable weakness and even those in the same area are not equally affected. Deep tendon reflexes tend to deteriorate with age and one-third of patients have been reported to experience predominating weakness of respiratory muscles. Patients may become wheelchair-bound and respiratory failure is the most common cause of death for this patient group. In adults, residual enzyme activity can be as much as 40% of normal (Chen and Amalfitano, 2000), which may explain the lack of cardiac involvement and the slow progression of disease. Glycogen accumulation in this patient group is mainly in skeletal muscles with little pathology evident in other tissues. However, variable glycogen accumulation has been reported in different muscle cells from the same patient (Hirschhorn and Reuser, 2001; Kishnani and Howell, 2004).

1.3.3 Diagnosis of GSD II

Diagnosis is usually made after clinical presentation with the onset of the characteristic pathological symptoms of the disease. Infantile-onset GSD II patients deteriorate rapidly and most survive only a few months after diagnosis (Van den Hout *et al.,* 2003). Older

children have more slowly progressing clinical symptoms and diagnosis can be delayed for years.

A sensitive but non-specific marker for GSD II is an elevation in serum creatine kinase level (Ausems *et al.,* 1999) with the greatest elevation found in infantile-onset patients; serum aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase increase significantly with age and appear to be good markers of disease progression (Van den Hout *et al.,* 2003). Other diagnostic techniques include electromyography and chest imaging studies (Hirschhorn and Reuser, 2001): the former shows the muscle weakness in most patients, although some muscles may appear to be normal in late-onset patients; the latter provides an indication of the massive cardiomegaly characteristic of infantile-onset patients but not the late-onset patients. A definitive diagnosis is by the absence (infantile) or reduced activity (juvenile and adult) of α-D-glucosidase using a fluorometric assay that measures the hydrolysis of natural or artificial substrates, including glycogen, the α-1,4-linked glucose disaccharide maltose and 4MU-α-D-glucopyranoside (Hirschhorn and Reuser, 2001). This enzyme assay can be performed on muscle, skin fibroblasts, lymphocytes, leucocytes and urine. Muscle and fibroblasts provide more reliable measures of α-D-glucosidase activity and are therefore primarily used (Hirschhorn and Reuser, 2001); A skin biopsy is less invasive and cell lines can be maintained for future use, however a muscle biopsy can yield faster results and can also provide additional information about glycogen storage within muscle cells. The major limitation with muscle biopsies in adult-onset GSD II patients is the great variability in pathology in different muscles and within muscle fibres.

Diagnostic tests that do not require a tissue biopsy are being developed, including measurement of glucose oligosaccharides or the total concentration of α-D-glucosidase protein and stored glycogen in, plasma, blood spots, and urine (An *et al.,* 2000; Umapathysivam *et al.,* 2001; Rozaklis *et al.,* 2002; Umapathysivam *et al.,* 2005). The measurement of acid α-D-glucosidase activity in dried blood spots with minimal interference by other enzymes that are active on the 4MU α-D-glucopyranoside artificial substrate has also been reported using maltose or acarbose as inhibitors (Chamoles *et al.,* 2004; Li *et al.,* 2004). Generally, infantile patients have undetectable enzyme activity, adult-onset patients exhibit considerable residual enzyme activity, and juvenile-onset patients exhibit an intermediate between the two. It should be noted, however, that considerable overlap is seen in residual enzyme activity between the clinical phenotypes (Hirschhorn and Reuser, 2001; Kishnani and Howell, 2004).

1.3.4 Genetics of GSD II

The α-D-glucosidase gene was localised to the small arm of human chromosome 17q25.2-q25.3 by fluorescence in situ hybridisation (Kuo *et al.,* 1996). The gene has been extensively characterised and shown to be 20 kb consisting of 20 exons (Hoefsloot *et al.,* 1990; Martinuik *et al.,* 1991; Tzall and Martinuik, 1991). There is extensive genetic heterogeneity for GSD II patients. The Erasmus Medical Centre in Rotterdam has catalogued (www.eur.nl/fgg/Chl/pompe.mutation.htm) more than 100 deleterious mutations in the α-D-glucosidase gene. The number of mutations is continually increasing with approximately 50% being missense mutations. The majority of missense mutations are located in highly conserved regions, including exon 11 (coding the catalytic site), exon 5 and exon 14. Nonsense, splice site and small frame-shift insertion/deletion mutations have also been reported. The majority of mutations are private, but a few are common

among ethnic groups: the leaky splice-site mutation IV 1-13t>g has an allele frequency of 0.4-0.6 in Caucasian adult-onset GSD II patients (Huie *et al.,* 1994); the missense mutation Asp645Glu has been shown to have a frequency of 0.8 in Chinese patients from Taiwan (Shieh and Lin, 1998); and the nonsense mutation Arg854X has a frequency of 0.5 in African-Americans (Adams *et al.,* 1997). Three mutations in the α-D-glucosidase gene are common in the Dutch patient population and include IV 1-13t>g, del525T, and delexon18 (Van der Kraan *et al.,* 1994). It was shown that 63% of Dutch GSD II patients carry one or two of these mutations and carrier screening in an unselected group of neonates for these mutations predicted the frequency of the disease to be 1 in 40, 000 (Ausems *et al.,* 1999). Another mutation reported to be common in the Dutch population is the missense mutation Gly309Arg (Kroos *et al.,* 1998).

1.3.5 Protein synthesis and post translational processing of α-D-glucosidase

The α-D-glucosidase cDNA is over 3.6 kb in length with 2856 nucleotides of coding sequence predicting a 952 amino acid protein of 105 kDa molecular mass (Hoefsloot *et al.,* 1988; Martiniuk *et al.,* 1990). α-D-Glucosidase is synthesised as a catalytically active precursor protein that is post-translationally modified by proteolysis, glycosylation and phosphorylation (Hoefsloot *et al.,* 1988; 1990; Wisselaar *et al.,* 1993). α-D-Glucosidase has a hydrophobic N-terminus where the signal peptide sequence resides. The signal peptide sequence is cleaved at the second potential cleavage site between glycine 28 and histidine 29, after which the α-D-glucosidase enzyme is termed the precursor form with a molecular mass of 110 kDa (Oude Elferink *et al.,* 1984; Tsuji and Suzuki, 1987). The α-D-glucosidase precursor is then proteolytically processed in late endosomes/lysosomes at both the N- and C-terminal ends, resulting in a number of mature forms, with the major species being 95, 76 and 70 kDa (Hoefsloot *et al.,* 1998; 1990; Wisselaar *et al.,* 1993).

The precursor α-D-glucosidase protein is extensively glycosylated and all seven potential glycosylation sites are used (Hermans *et al.,* 1993). It also has an intracellular half-life of only two-hours reflecting traffic to lysosome and subsequent processing. In contrast the 76 and 70 kDa mature α-D-glucosidase forms have a half life of five- to eight-days (Hirschhorn and Reuser, 2001). The mature forms of α-D-glucosidase have a seven-fold increase in glycogen activity compared to the 110 kDa precursor, and the 95 kDa form has an intermediate value (Wisselaar *et al.,* 1993). The need for proteolytic processing for optimal activity of the natural substrate glycogen could relate to a conformational change that would allow the large substrate glycogen better access to the catalytic site (Hirschhorn and Reuser, 2001).

1.4 MPS I

Glycosaminoglycans are long unbranched polysaccharides made up of repeating disaccharides that may be sulphated and form an important component of connective tissues. α-L-Iduronidase (EC 3.2.1.76) specifically cleaves α-linked iduronic acid residues from the non-reducing ends of the mucopolysaccharide substrates derived from the glycosaminoglycans dermatan sulphate (DS) and heparan sulphate (HS) (Figure 1.6) (Neufeld and Muenzer, 2001). MPS I results from the functional deficiency of the lysosomal glycosidase α-L-iduronidase, which prevents the sequential catabolism of HS and DS. These undegraded substrates consequently accumulate within the lysosomes of affected cells and are also excreted in the urine. The first clinical description of a patient with a mucopolysaccharidoses (MPS) was made in 1907 by Berkhan and he described clinical symptoms consistent with MPS I (cited in Neufeld and Muenzer, 2001). This was followed by a more detailed description by Gertrud Hurler and Meinhard von Pfaundler in 1919, of two patients with a condition referred to as Hurler-Pfaundler syndrome, now

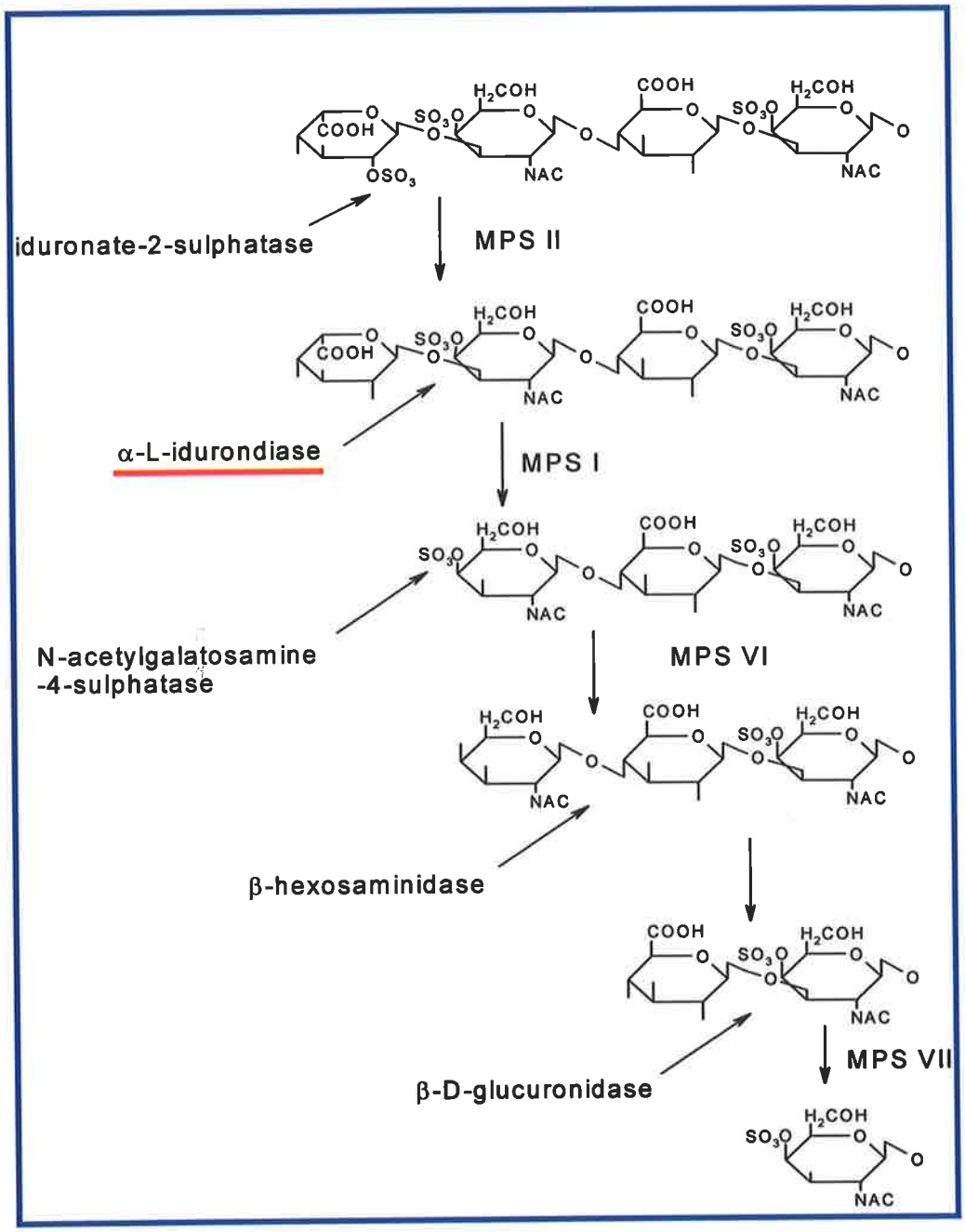

Figure 1.6: α-L-Iduronidase activity

α-L-Iduronidase hydrolyses iduronic residues from the non-reducing end of the mucopolysaccharide substrate, dermatan sulphate. Adapted from Neufeld and Muenzer, 2001.

known as Hurler syndrome (cited in Neufeld and Muenzer, 2001). Initially proposed to involve altered lipid metabolism, the disorder was described as a lipochondrodystrophy, but this was corrected in 1952 by Brante who used the term 'mucopolysaccharidosis' and classified the group of MPS disorders according to chemical analysis and inclusion body staining. Dorfman and Lorincz (1957) were the first to show that affected patients excreted mucopolysaccharides to excess in urine; Danes and Bearn (1965) demonstrated mucopolysaccharide storage in cultured fibroblasts.

1.4.1 Clinical phenotype of MPS I

A continuous spectrum of clinical phenotype exists for MPS I but three clinical phenotypes representing different degrees of severity have been reported in the literature: Hurler, Scheie and Hurler-Scheie (Roubicek, 1985; Hopwood and Morris, 1990; Neufeld and Muenzer, 2001). Scheie syndrome was first recognised in 1962 (Scheie *et al.,* 1962) and was later shown to result from the same enzyme deficiency as Hurler syndrome (Wiesmann and Neufeld, 1970).

1.4.1.1 Hurler syndrome

Hurler syndrome represents the rapidly progressing end of the MPS I clinical spectrum. Patients present in the first two-years of life with rapidly progressing clinical symptoms, including mental retardation, hydrocephalus, short stature, coarse facial features, skeletal deformity, clawed hands, spinal compression, enlarged tongue, cardiac disease, corneal clouding, deafness, sleep apnoea, persistent nasal discharge, joint deformity and stiffness, frequent infections, fatigue and hepatosplenomegaly (Figure 1.7a). Diagnosis is usually made between four- and 18-months and untreated patients generally die within the first decade of life (Neufeld and Muenzer, 2001).

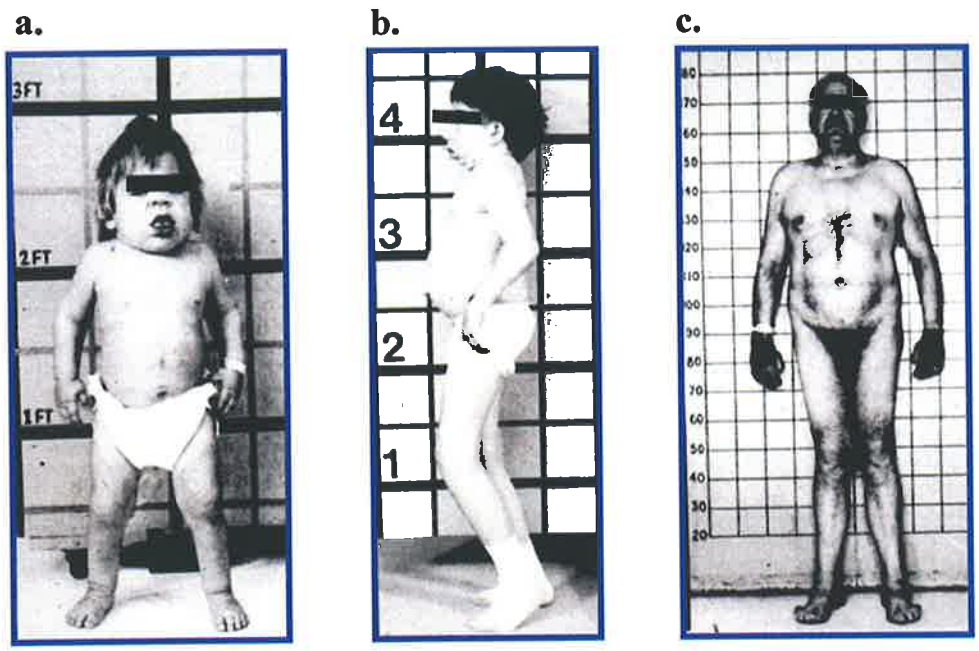

Figure 1.7: MPS I clinical phenotype

Three clinical phenotypes of MPS I representing different degrees of severity. Adapted from Neufeld and Muenzer, 2001.

1.4.1.2 Hurler-Scheie syndrome

Patients with Hurler-Scheie syndrome have an intermediate clinical phenotype, generally display clinical symptoms between three to eight years of age and tend to have a longer life-span than Hurler patients. Symptoms may include some mental retardation, clawed hands, hepatosplenomegaly, obstructive airway disease, skeletal deformities, joint deformity and stiffness, short stature, coarse facial features, deafness, cardiac disease and fatigue (Fig 1.7b). Death in Hurler-Scheie patients typically occurs in the late-teenage years (Neufeld and Muenzer, 2001).

1.4.1.3 Scheie syndrome

Scheie syndrome represents the slowly progressing end of the MPS I clinical spectrum: the onset of pathology is delayed, disease progression is slower and the presentation of clinical signs more variable. Clinical symptoms include hernia, slight hepatosplenomegaly, corneal clouding, obstructive airway disease, clawed hands, cardiac disease, joint deformity and joint stiffness (Figure 1.7c). Scheie patients usually have little or no neuronal involvement and can often have normal stature and life expectancy. The onset of significant symptoms usually occurs after the age of five-years but diagnosis commonly occurs between 10- and 20-years of age (Neufeld and Muenzer, 2001).

1.4.2 Genetics of MPS I

The α-L-iduronidase gene was localised to chromosome 4p16.3 by in situ hybridisation and Southern blot analysis of mouse-human cell hybrids (Scott *et al.,* 1990). The α-L-iduronidase cDNA was first isolated and expressed in 1991 (Scott *et al.,* 1991). Scott and colleagues (1992) demonstrated that the α-L-iduronidase gene spans approximately 19 kb and contains 14 exons. Over 90 disease-causing α-L-iduronidase gene mutations have been

reported in the Human Gene Mutation Database (HGMD, www.hgmd.org). The two most common mutations in Caucasians are W402X and Q70X accounting for up to 70 percent of the α-L-iduronidase disease alleles in some countries, (Bunge *et al.*, 1994; 1995; Scott *et al.*, 1995; Gort *et al.*, 1998; Beesley *et al.*, 2001). These two mutations introduce premature stop codons that consequently lead to no detectable α-L-iduronidase protein (Ashton *et al.*, 1992; Hein *et al.*, 2004) and have therefore been described as "null alleles". Consequently, these patients have a rapidly progressing clinical presentation (Hurler syndrome) due to the absence of α-L-iduronidase protein and activity. Premature stop codon mutations are more frequent in MPS I patients when compared to other MPS disorders, with a total of 15 identified to date (Beesley *et al.*, 2001; Hein *et al.*, 2004). There is potential of natural read-through at the premature stop codon mutation and the production of small amounts of normal α-L-iduronidase protein. The level of read-through was shown to be dependant on the specific stop codon and its surrounding sequence (Hein *et al.*, 2004). The R628X and the W180X stop codon mutations produced a detectable level (0.2-0.4 pmol/min/mg) of natural stop codon read-through in two MPS I patient skin fibroblasts (Hein *et al.*, 2004). This was consistent with the low fidelity TGA stop codons in these MPS I Patients. However high fidelity TAG stop codons (Q70X, Y343X, Q400X and W402X mutations) show very little read-through potential and skin fibroblasts from these patients had no detectable α-L-iduronidase activity (Hein *et al.*, 2004). Several other mutations abolish α-L-iduronidase activity and are associated with a rapidly progressing clinical phenotype. These include D349N, E182A and E299A, which have been shown to result in relatively normal amounts of α-L-iduronidase protein that is devoid of activity (Brooks *et al.*, 1992; 2001). The missense mutation, A75T, also results in residual mutant α-L-iduronidase protein with no detectable enzyme activity (Clarke *et al.*, 1994; Beesley *et al.*, 2001).

A total of 61 α-L-iduronidase missense/nonsense gene mutations have been reported to date, with the majority resulting in low levels of α-L-iduronidase protein and residual α-L-iduronidase activity (HGMD). The α-L-iduronidase mutation P533R has a high frequency in MPS I patients of Moroccan and Sicilian-Italian origin (Alif *et al.,* 2000; Gatti *et al.,* 1997 respectively). Patients homozygous for the P533R mutation show a slightly less progressive clinical phenotype, compared to classical Hurler syndrome patients. Two mutations common in Japanese MPS I patients include the insertion nt704ins5, and the missense mutation R89Q, which result in slowly progressing clinical phenotypes (Yamagishi *et al.,* 1996).

1.4.3 Diagnosis of MPS I

Following clinical suspicion, analysis of the urine for the presence of glycosaminoglycans, by high-resolution electrophoresis acts as a preliminary screen (Hopwood and Harrison, 1982) for MPS disorders. This test provides an indication of a patient's likely MPS type based on the presence of undegraded substrate (e.g. DS and HS for MPS I). The definitive diagnosis of MPS I relies on demonstrating a deficiency of the lysosomal enzyme α-L-iduronidase using specific enzyme assays (Hopwood and Muller, 1982; Ashton *et al.,* 1992). Enzyme activity may be measured in most tissues, but diagnosis is usually made in leucocytes, cultured skin fibroblast cell extracts or serum (Neufeld and Muenzer, 2001; Hopwood and Morris, 1990). Mutant protein levels and enzyme kinetics can then be determined on fibroblast cell extracts to provide a measure of enzyme catalytic capacity (Ashton *et al.,* 1992), which gives an indication of the patient's expected clinical phenotype (Yogalingam *et al.,* 2004). Together with enzyme activity and protein assays, molecular genetic analysis of patient DNA can be conducted, collectively providing a

reasonably accurate prediction of the clinical phenotype for most MPS I patients (Yogalingam *et al.,* 2004).

1.4.4 Protein synthesis and post-translational processing of α-L-iduronidase

Scott *et al.,* (1991) reported the full-length protein sequence of α-L-iduronidase, predicting a 653 amino acid polypeptide. Northern blot analysis demonstrated a single mRNA species of 2.3 kb in human kidney. α-L-Iduronidase was shown to have a 26 amino acid signal peptide, which is cleaved in the endoplasmic reticulum and results in the 74 kDa polypeptide, present in human liver. Further studies indicated a diverse composition of α-L-iduronidase polypeptide species of the mature lysosomal forms, with at least seven different species reported (74, 65, 60, 49, 44, 18 and 13 kDa) (Clements *et al.,* 1989; Scott *et al.,* 1991; Taylor *et al.,* 1991). α-L-Iduronidase secreted from human fibroblasts was shown to have a higher molecular mass of 81 kDa, suggesting that intracellular proteolytic processing of α-L-iduronidase occurred within the endosome-lysosome compartments (Taylor *et al.,* 1991). All six potential N-glycosylation sites on α-L-iduronidase can be utilised (Neufeld and Muenzer, 2001).

1.5 Therapy for LSD

The concept that LSD could be treated by replacing the defective enzyme with normal enzyme was first suggested in 1964 by De Duve following the discovery of the lysosome and that LSD were caused by deficiencies in lysosomal enzymes (Hers, cited in Hirschhorn and Reuser, 2001). This provided the rationale behind the development of the first specific therapies that address the enzyme deficiency, including bone marrow transplantation (BMT) and ERT. Several other specific therapies for LSD are either in clinical practice or in development, and include, substrate deprivation therapy (SDT), enzyme enhancement

therapy (EET), gene replacement therapy (GRT), stop codon read-through therapy, neo-organ therapy and cell-based therapies.

BMT in LSD is designed to reconstitute the patient's haematopoietic system with stem cells from bone marrow (or sometimes cord blood) of an immune compatible healthy donor (Neufeld and Muenzer, 2001). The donor's circulating mononuclear leukocytes can integrate into many tissues and consequently can become a life-long source of enzyme. The first BMT for a LSD was carried out in the early 1980s on a MPS I patient that was thought at time to have a Hurler phenotype (Hobbs *et al.,* 1981). This patient was later shown to have a more slowly progressive form of MPS I (Hopwood *et al.,* 1993). Subsequently, two Hurler patients homozygous for the W402X mutation were treated by BMT and it significantly slowed down the clinical regression ten years post treatment (Hopwood *et al.,* 1993). Since then BMT has been carried out for at least 20 LSD types (Vellodi, 2005). Although BMT has been effective in correcting somatic pathology and CNS pathology to variable degrees for a number of LSD, it does not always result in complete reversal of the clinical phenotype (Wilcox, 2004). Furthermore, skeletal deformation, joint stiffness and ocular anomalies are not always corrected by BMT and patients can require extensive corrective surgery (Field *et al.,* 1994; Gullingsrud *et al.,* 1998).

The clinical outcome of BMT in LSD patients depends on the extent of pathology and age at the time of transplantation. Experience has highlighted the importance of performing BMT as early as possible to maximise success (e.g. engraftment and enzyme production). However, this therapeutic approach is still associated with major problems and risks, including the difficulty of finding a compatible donor, a transplant failure rate of 10-15 %,

a mortality rate of 10-20 % and risk of graft-versus-host disease (Whitley *et al.,* 1993; Wilcox, 2004). Disease-related risks prior to BMT and complications that can compromise the success of this procedure, such as pneumonia, airway obstruction, poor cardiac function and hydrocephalus are issues that also need to be addressed to maximise patient survival. Due to the associated risks, BMT is generally only used to treat LSD patients with rapidly progressing pathology with CNS involvement who have been diagnosed prior to the onset of deleterious symptoms. An alternative to BMT that has been recently evaluated is the transplantation of umbilical cord blood from unrelated donors. Sustained engraftment was observed for transplanted asymptomatic patients with Hurler's syndrome and infantile Krabbe's disease (Staba *et al.,* 2004; Escolar *et al.,* 2005 respectively). Transplantation also improved neurocognitive performance and decreased somatic pathology in these patients. Some of the benefits of umbilical cord blood grafts over BMT include rapid availability of a donor graft and even reduced risk of growth versus host disease.

Gene therapy holds great promise for treating LSD by supplying a normal copy of the gene for the defective protein, which then provides the patient with an endogenous source of enzyme. Two different approaches to gene therapy are being developed for LSD: the first involves the direct administration of recombinant vectors in target tissues, such as the CNS or liver (Yew and Cheng, 2001); the other approach involves genetically altering cells (fibroblasts, progenitor cells, muscular cells) to produce the missing enzyme and then returning them to the patient (Wilcox, 2004). For significant clinical benefit in LSD patients, both of these gene therapy strategies must result in significant enzyme secretion from the corrected cells to enable the correction of other cells or tissues. Although the feasibility of gene therapy has been demonstrated in a number of LSD animal models and

considerable advances have been made in this field, GRT is still in its infancy and many critical issues require to be investigation, including adequate and sustained expression of the gene in the necessary cell type or tissue, vector design and safety concerns (Poenaru, 2000).

Unlike the other therapeutic options for LSD that replenish the deficient enzyme, SDT aims to reduce substrate influx into the lysosomes by inhibiting substrate biosynthesis. In theory, SDT would sufficiently slow down substrate accumulation to levels below the toxic threshold and consequently patients would be asymptomatic or exhibit a greatly reduced rate of disease progression (Butters *et al.,* 2003). To date, SDT has been utilised to treat LSD involving glucose-based glycosphingolipid accumulation (e.g. Gaucher, Fabry, Tay-Sachs, Sandhoff diseases etc) by using inhibitors that act on the first step of the biosynthesis of glycosphingolipids. The substances used for substrate deprivation are able to pass through blood–brain barrier since they are small. In animal models of Tay-Sachs and Sandhoff diseases, SDT using the imino sugar inhibitor N-butyldeoxynojirimycin (NB-DNJ), resulted in an increase in life expectancy, a reduction in substrate accumulation in the CNS, delayed onset of symptoms and delayed disease progression (Platt *et al.,* 1997; Jeyakumar *et al.,* 1999 respectively). A clinical trial of SDT in type I Gaucher disease patients showed effective depletion of glycosphingolipids and improvement in several clinical parameters (Cox *et al.,* 2000). Further studies of NB-DNJ (Miglustat, Zavesca^{TM}) in Niemann-Pick disease type C, late-onset Tay-Sachs disease and type III Gaucher disease are in progress. Diarrhoea has been reported to be a dose-limiting side effect of NB-DNJ treatment and one that generally leads to therapeutic non-compliance, but preclinical trials using a galactose analogue N-butyldeoxygalactonojirimycin (NB-DGJ) indicate better tolerance (Andersson *et al.,* 2004). Since it is unlikely that NB-DNJ will be effective in all

the sphingolipid disorders, the identification of other therapeutic agents will be required. Challenges that lie ahead for SDT include the identification of inhibitors that are well tolerated by humans and optimal doses to correct pathology without compromising cellular structure and function, which are key roles of the substrates being inhibited.

Another approach to LSD treatment that has generated considerable interest is EET (otherwise known as chemical chaperone therapy). A number of mutations in LSD patients result in mis-folded or mis-trafficked lysosomal enzymes (Brooks, 1997). Mis-folded proteins are retained in the endoplasmic reticulum where they are identified by the cell's quality control system, the endoplasmic reticulum-associated degradation pathway, and undergo rapid degradation (Ellgaard and Helenius, 2001; Jarosch *et al.,* 2003). However, in some cases, the active site may fold normally and the enzyme retains some catalytic activity. The stabilisation of mis-folded proteins can be enhanced by EET, using chemical chaperones such as substrate analogues, receptor agonists and antagonists or other modulators. These low-molecular weight chemical chaperones can specifically and reversibly bind to and rescue mis-folded or mis-targeted protein and consequently increase the mutant protein function and clinical benefit (Desnick, 2004). EET has recently attracted considerable interest, especially as it has been shown that chaperones are capable of crossing the blood-brain barrier and may have potential in treating the CNS pathology in some LSD. Preclinical trials of EET in Gaucher disease/β-glucosidase (Sawkar *et al.,* 2002), mucopolysaccharidosis type IV (MPS IVB)/β-galactosidase (Matsuda *et al.,* 2003), Fabry disease/α-D-galactosidase A (Fan *et al.,* 1999) and Tay-Sachs and Sandhoff diseases/β-hexosaminidase (Tropak *et al.,* 2004), have shown promising results. A major limitation of EET is the requirement for residual enzyme activity for the chaperone to be effective.

ERT is currently the most viable therapeutic option available for many LSD patients and is already in clinical practice for Gaucher, Fabry, MPS I and more recently MPS VI patients. ERT was first proposed by De Duve (1964) and involves exogenous administration of the missing enzyme (Figure 1.1). A key step towards the development of ERT was the cross-correction of enzyme activity in MPS I (and MPS II) fibroblasts, where a diffusible factor (later shown to be α-L-iduronidase for MPS I) corrected storage by uptake into affected cells (Frantantoni *et al.,* 1968). Furthermore, the correction of LSD patient fibroblasts by the addition of enzyme derived from other sources provided the logical basis for exogenous enzyme administration (Di Ferrante *et al.,* 1973; O'Brien *et al.,* 1973; Cantz and Kresse, 1974). However, early attempts at enzyme replacement were ineffective because of the inability to obtain sufficient amounts of purified enzyme and a poor understanding of receptor-mediated uptake of lysosomal enzymes. It is now known that mannose-6-phosphate receptors at the cell surface bind the mannose-6-phophate residues on lysosomal enzymes and facilitate the delivery of the enzyme-receptor complex to the late endosome/lysosome via the endocytic network (Kornfeld, 1987; Figure 1.1). In this way, exogenously administered active enzyme can reach the target organelle, correct the deficiency and consequently alleviate disease progression.

The use of ERT to treat LSD has several major limitations and potential risks. The first is the inability of the large molecules, such as the administered enzyme, to cross the adult blood-brain barrier to treat CNS pathology, a clinical manifestation characteristic in a number of LSD. Recent evidence suggested that the mannose-6-phosphate receptor was developmentally regulated and declined in most tissues (including the brain) of mouse during the postnatal period (Urayama *et al.,* 2004). This would explain why adult CNS is resistant to ERT. Identifying ways to induce the expression of the mannose-6-phosphate

receptor in the adult blood-brain barrier would therefore have great therapeutic benefits. Another limitation of ERT is the need to target enzyme uptake to specific somatic cell types or tissues; for example, targeting α-D-glucosidase to the major site of pathology in GSD II patients, skeletal muscle, has been problematic (Raben *et al.,* 2003; Klinge *et al.,* 2005). A potential risk of ERT in LSD patients is the development of antibodies to the exogenously administered enzyme. The effect of antibodies on ERT in LSD will be the focus of discussion in section 1.6 and this thesis. Despite these limitations, ERT is currently the most clinically viable therapy for LSD with non-neuronal pathology; the following sections will focus on *in vitro* studies, preclinal and clinical trials of this strategy for MPS I and GSD II patients.

1.5.1 *In vitro* studies of ERT in MPS I and GSD II

Initially, lysosomal enzymes were purified from tissue sources, which did not prove to be ideal for ERT applications. The low abundance of α-D-glucosidase from urine made it an impractical source and α-D-glucosidase from human placenta lacked the necessary mannose-6-phosphate residues for receptor-mediated uptake (Hirschhorn and Reuser, 2001). Similarly, initial attempts to purify α-L-iduronidase resulted in partially purified preparations from urine, kidney, liver and lung that were unsuitable for use in therapy (Neufeld and Muenzer, 2001).

The cloning of the respective genes prompted investigations into the development of expression systems for the production of recombinant lysosomal enzymes. Following the initial expression of full-length α-L-iduronidase and α-D-glucosidase cDNA in Chinese hamster ovary cells (CHO-K1) (Scott *et al.,* 1991; Unger *et al.,* 1994; Van Hove *et al.,* 1996; Fuller *et al.,* 1995), as well as other high expression systems, large increases in the

amount of secreted enzyme were reported that would accommodate the high demand for enzyme in ERT studies. Furthermore, these studies demonstrated that the secreted α-L-iduronidase and α-D-glucosidase could respectively correct the lysosomal storage present in MPS I and GSD II skin fibroblast cells *in vitro* (Unger *et al.,* 1994; Van Hove *et al.,* 1996 respectively). Inhibition of α-L-iduronidase and α-D-glucosidase uptake was achieved by the addition of mannose-6-phosphate in the culture medium, highlighting the importance of the mannose-6-phosphate receptor-mediated uptake of these two lysosomal enzymes (Bach *et al.,* 1972; Fuller *et al.,* 1995).

1.5.2 Preclinical and clinical trials of ERT in GSD II

Following proof-of-principle for ERT, evaluation in preclinical and clinical trials commenced to establish both the potential efficacy of the administered enzyme for the clearance of stored substrate and the safety associated with the infusion of recombinant enzyme. For GSD II, ERT has been shown to be effective in the Japanese quail and the GSD II mouse models (Kikuchi *et al.,* 1998; Bijvoet *et al.,* 1998; 1999; Raben *et al.,* 2003). In these studies, ERT corrected the deficiency and reduced glycogen storage, primarily in the heart and to variable degrees in the skeletal muscle. These studies also indicated that potentially large doses of enzyme could be required for efficient ERT in GSD II patients.

Two phase I/II clinical trials of ERT were initiated on infantile-onset GSD II patients using α-D-glucosidase purified from two different sources: over-expressing CHO-K1 cells (Fuller *et al.,* 1995; Van Hove *et al.,* 1996) and milk from transgenic rabbits (Bijvoet *et al.,* 1999). Enzyme doses utilised in these trials were, respectively 5 mg/kg (Amalfitano *et al.,* 2001) and 20-40 mg/kg (Van den Hout *et al.,* 2000). In both trials the greatest effect of

ERT was reported in the heart, with marked reductions in size and improved cardiac function observed throughout the course of therapy. Significant improvements in motor function were also seen. Muscle biopsies revealed normalisation of α-D-glucosidase activity but no significant change in total tissue glycogen. Muscle biopsies also showed large areas with virtually normal muscle morphology, but not all muscle fibres were equally affected in the same patient. Treated patients passed the typical mean age of one-year of survival for infantile-onset GSD II patients and reached milestones that untreated patients do not reach, including learning to sit up-right without support, crawl, walk and climb stairs. The best improvements in motor function were seen in patients who started ERT at an early age, prior to irreversible disease progression (Winkel *et al.,* 2003; Van den Hout *et al.,* 2004). All patients developed immunoglobulin G antibodies against the exogenously administered α-D-glucosidase enzyme.

The two clinical trials indicated differences in the dose of recombinant enzyme utilised: the study using recombinant human (rh-) α-D-glucosidase purified from transgenic rabbits utilised an eight-times higher concentration compared to the enzyme purified from CHO-K1 cells. A possible explanation for this difference may be variations in glycosylation and the level of accessible mannose-6-phosphate residues on the different rh-α-D-glucosidase enzymes. It is still unclear as to why skeletal muscle is cleared less efficiently than the heart, but this may relate to differences in enzyme accessibility. Skeletal muscle has been reported to have a lower abundance of mannose-6-phosphate receptors than heart tissue (Wenk *et al.*, 1991; Funk *et al.,* 1992; Raben *et al.,* 2003) and the rh-α-D-glucosidase has been shown to have less than optimal affinity for the mannose-6-phosphate receptor (Zhu *et al.,* 2004). This might offer another explanation for the high dosage required for enzyme correction in GSD II compared to other LSD treated by ERT. Recently, it was

demonstrated that chemical conjugation onto rh-α-D-glucosidase of a synthetic oligosaccharide ligand bearing mannose-6-phosphate residues in the optimal configuration for binding the receptor improved its affinity and resulted in greater clearance of glycogen in muscles of GSD II mice when compared to the unmodified rh-α-D-glucosidase (Zhu *et al.,* 2005). This could be a feasible approach to enhance the efficacy of ERT in GSD II patients but may also facilitate a reduction in the enzyme doses required to effectively treat these patients.

To test the efficacy of ERT in juvenile- and adult-onset GSD II patients, a phase I/II open label trial was initiated with weekly infusions of 20 mg/kg of rh-α-D-glucosidase purified from transgenic rabbit milk (Winkel *et al.,* 2004). After three-years of therapy all patients had stabilised pulmonary function and reported less fatigue. The effect of treatment was most significant in the youngest and least affected patient. This patient, who had been wheelchair-dependent for four-years, gained normal muscle strength and function and started to walk. All patients developed immunoglobulin G antibodies against the exogenously administered rh-α-D-glucosidase enzyme.

The success of the phase I/II clinical trials using rh-α-D-glucosidase from the two sources led to the initiation of two phase II open-label, multicentre trials in infantile-onset patients. Similar efficacy and safety to that described in the phase I/II trials were reported (Desnick, 2004; Kishnani and Howell, 2004; Klinge *et al.,* 2005). Genzyme Corporation is currently conducting a phase III trial using rh-α-D-glucosidase from CHO-K1 cells (Myozyme®, www.genzyme.com). The high enzyme doses required for therapeutic effect and the variability in glycogen clearance in the different affected tissues are clearly issues that

need to be further addressed in order to provide an efficient and cost-effective ERT strategy for this disorder.

1.5.3 Preclinical and clinical trials of ERT in MPS I

Preclinical studies of ERT for MPS I demonstrated the efficacy of rh-α-L-iduronidase for the clearance of glycosaminoglycans (Shull *et al.,* 1994; Clarke *et al.,* 1997; Kakkis *et al.,* 2001). On the basis of these preclinical studies an open-label phase I clinical trial was conducted in 10 MPS I patients (Kakkis *et al.,* 2001) using rh α-L-iduronidase produced in CHO-K1 cells (Aldurazyme®, jointly produced by BioMarin Pharmaceutical Inc. and Genzyme Corporation). Of the 10 patients, nine displayed a Hurler-Scheie clinical phenotype and only one patient had Hurler phenotype. All patients were infused weekly with rh-α-L-iduronidase (0.58 mg/kg body weight) for a duration of 104-weeks (Kakkis *et al.,* 2001; Wraith, 2001). Treatment efficacy was measured by a reduction of either spleen or liver size and a reduction in urinary glycosaminoglycans. Hepatosplenomegaly decreased significantly over the course of treatment in all patients and by 26-weeks, eight of the patients had normal body liver size for body weight and age. Other improvements reported during this clinical trial included an increase in growth and height, improved heart function, a reduction in sleep apnoea and hypopnea and an improvement in general physical function. Adverse events were reported, including infusion-associated reactions.

A phase III multicentre, randomised, double blind, placebo-controlled clinical trial was initiated involving 45 MPS I patients (Wraith *et al.*, 2004). The majority of enrolled patients (82%) were defined as having MPS I of intermediate severity, Hurler-Scheie syndrome. Statistically significant increases in forced vital capacity ($p=0.016$) and in endurance as measured by a six-minute walk test ($p=0.066$) were reported for MPS I

patients treated with rh-α-L-iduronidase, when compared to the placebo controls. Reductions in liver size, urinary glycosaminoglycan levels and significant improvements in patients with sleep apnoea and hypopnea were also reported compared to the placebo controls. The findings in this trial corresponded with the findings of the earlier phase I/II clinical trial. The safety profile was comparable between the two treatment groups, supporting rh-α-L-iduronidase ERT as a safe treatment strategy for MPS I patients. Infusion-associated reactions were common and most patients developed immunoglobulin G to the recombinant enzyme. All 45 patients completed the trial and continued treatment in an open-label phase III extension study. Aldurazyme was recently approved by the US Food and Drug Administration for clinical use (Aldurazyme®, 2003).

1.6 Immune response to ERT

An immune response presents as the only major complication to ERT in LSD patients. The first immune-mediated complication is the possible development of hypersensitivity reactions during or immediately after administration of enzyme. The second potential complication involves the effect of circulating antibody to the replacement protein. These will be discussed in greater detail in the sections to follow.

1.6.1 Measures of an immune response

A number of factors are indicative of an immune response and these include clinical signs, increased levels of circulating antibodies against the protein in question and complement activation. A direct measure of humoral immune response is the presence of circulating antibodies against the infused protein. An enzyme-linked immunosorbent assay (ELISA) can be used to detect the presence of immunoglobulin G and immunoglobulin E antibodies to the infused enzyme and quantify the extent of the response. The antibody titres detected

by ELISA can be confirmed by radioimmunoprecipitation analysis, which involves the detection of antibodies by the use of radiolabeled antigen. Further characterisation of the immune response can be achieved by determining the type of epitope the antibodies recognise.

Complement can be activated by interaction with antibody-antigen complexes of the immune system and constitutes a group of serum proteins involved inflammation, the activation of phagocytes and the lytic attack on cell membranes (Walport, 1998). The detection of complement activation is usually achieved by the presence of degradation products in the serum of one of the complement proteins, C3. Two-dimensional immunoelectrophoresis can be used to detect C3 degradation products, showing the conversion of one complement peak to two peaks. The level of serum tryptase is also an indicator of immune reaction as it is a protease secreted by activated mast cells of the immune system.

1.6.2 Frequency of immune response in ERT-treated LSD animals and patients

Immune response to ERT has been reported in LSD animal models. In an MPS VI cat model that has some residual 4-sulphatase activity, antibodies to rh-4-sulphatase were detected in 30% of the treated animals (Brooks *et al.,* 1997). However, MPS VI cats treated from birth developed negligible antibody titres to the administered rh-4-sulphatase protein (Auclair *et al.,* 2003) suggesting the development of antibodies could be prevented by early treatment when the immune system is more amendable to tolerance induction. Antibody responses to the replacement protein were also reported in 100% of MPS I dogs (null mutation, no protein), treated with recombinant α-L-iduronidase, (Shull *et al.,* 1994;

Kakkis *et al.,* 1996) and 100% of GSD II mice (a knockout model, no protein) treated with rh-α-D-glucosidase (Raben *et al.,* 2003).

Antibody responses have been reported in variable numbers of ERT-treated patients as summarised in Table 1.2: in Gaucher disease patients receiving rh-β-glucocerebrosidase (EC 3.2.1.45, Cerezyme®), 142 (13%) of 1122 patients demonstrated an immune response to the infused protein (Rosenberg *et al.,* 1999; Cerezyme®, 2002); in MPS I, 50 (91%) of 55 patients have been reported to develop antibodies to rh-α-L-iduronidase (EC 3.2.1.76, Aldurazyme®, 2003). In infantile-onset GSD II patients treated with rh-α-D-glucosidase (EC 3.2.1.20) derived from CHO culture, 14 (83%) of 18 patients developed antibodies against the replacement protein (http://www.worldpompe.org/myozyme260405.html). To date all patients treated with rh-α-D-glucosidase from transgenic rabbit milk developed antibodies against the replacement enzyme during the first 20- to 48-weeks of therapy (Van den Hout *et al.,* 2004; Winkel *et al.,* 2004). In a phase I/II open label extension study in Fabry patients receiving rh-α-D-galactosidase A purified from CHO-K1 cell line (EC 3.2.1.22, Fabrazyme®), 51 (88%) of 58 patients developed antibody titres to the infused protein (Eng *et al.,* 2001; Fabrazyme®, 2003). A further clinical trial in Fabry patients resulted in 30 (55%) of 55 patients developing antibody titres to rh-α-D-galactosidase A purified from a human cell line (Replagal®, 2001). In MPS VI patients receiving rh-*N*-acetylgalactosamine 4-sulphatase (EC 3.1.6.12, Naglazyme™), 53 (98%) of 54 patients showed evidence of an antibody response to this replacement protein (Naglazyme™, 2005).

Table 1.2: Humoral immune response to ERT in LSD

DISEASE	ENZYME	THERAPY STATUS	% OF PATIENTS WITH ANTIBODY REACTIVITY
Gaucher	Cerezyme®	Clinical practice	15
MPS I	Aldurazyme ®	Clinical practice	91
MPS II	Iduronate-2-sulphatase	Phase III	11
MPS VI	Naglazyme™	Clinical Practice	98
Fabry	Fabrazyme ® Replagal ®	Clinical practice	89 55
GSD II	α-glucosidase Myozyme ®	Phase I/II Phase II/III	66 83

Adapted from Brooks *et al.*, 2003.

1.6.3 Adverse effects of antibody production

Adverse events during or immediately after infusion of enzyme are indicative of a hypersensitivity reaction to the infused protein. Patients can experience a combination of symptoms, which can be life-threatening and are therefore an important concern. In Gaucher patients, adverse events have been detected in ~13.8% of patients receiving intravenous infusion of Cerezyme® (Cerezyme®, 2002). Reactions included pruritus, burning, swelling or sterile abscess at the infusion site, nausea, abdominal pain, vomiting, diarrhoea, rash, fatigue, headache, fever, dizziness, chills, backache, tachycardia, flushing, urticaria/angioedema, chest discomfort, respiratory symptoms, cyanosis and hypotension. These reactions occurred early in the treatment period and had a tendency to reduce after a higher number of enzyme administrations. However, a few Gaucher patients were reported to develop a later-onset immune response. Anaphylactic reactions have been reported in <1% of Gaucher patients treated with ERT and, due to the life-threatening nature of the adverse response in these patients, extreme caution is required for further enzyme administration. Slower infusion rates and premedication with antihistamines and/or corticosteroids have been shown to be effective in reducing the clinical signs of hypersensitivity reactions.

Similar adverse reactions have been reported in other LSD patients receiving ERT. In Fabry patients, ≥10% of patients treated with either Fabrazyme® or Replagal® demonstrated signs of hypersensitivity-type reactions (Replagal®, 2001, Fabrazyme®, 2003). In the MPS I clinical trials, hypersensitivity-type reactions were common: one patient who tested positive for immunoglobulin G antibodies and complement activation had an anaphylactic reaction requiring emergency procedures (Aldurazyme®, 2003). Five patients were reported with transient urticaria during enzyme infusion (accompanied by

angioedema and transient hypoxemia in some cases) and consequently were either infused at a reduced rate or temporarily infused with a reduced enzyme dose. Hypersensitivity reactions were also controlled by increased doses of prophylactic drugs, such as diphenhydramine, either before or in some cases during enzyme infusion. The frequency of these hypersensitivity-type reactions reduced during the study and eventually stopped in all patients. Four patients showed biochemical evidence of complement activation early in the study, but by 26-weeks it was no longer apparent. The majority of patients in the rh- α-L-iduronidase phase III clinical trial developed immunoglobulin G antibodies. Although a number of adverse reactions were reported, a similar number were reported for both the enzyme treatment and the placebo control groups.

In a phase II clinical trial involving MPS VI patients infused with Aryplase®, five adverse events were reported during infusion and eight events reported during the day of infusion out of a total of 240 infusions (Aryplase®, 2003). The most common hypersensitivity-type reactions included abdominal pain, febrile reactions and pruritus. However, of the seven serious adverse events, only one was possibly related to the drug.

Hypersensitivity reactions have also been reported in infantile-onset and late-onset GSD II patients treated with rh-α-D-glucosidase from transgenic rabbit milk (van den Hout *et al.*, 2004; Winkel *et al.*, 2004; Klinge *et al.*, 2005) and from CHO-K1 cells (Amalfitano *et al.*, 2001; Kishnani and Howell, 2004). Hypersensitivity-type reactions generally occurred during the initial phase of treatment, and commonly included fever, malaise, erythematous rash, sweating, hypoxia, flushing and tachycardia.

Strategies to control these adverse reactions included reducing the rate of enzyme infusion and co-administration of anti-inflammatory drugs. It is also important to appreciate that not all of the observed adverse reactions are immune-mediated; in fact some adverse events appear to be idiosyncratic, having also been reported in some placebo control groups (Eng *et al.,* 2001; Aldurazyme®, 2003; Fabrazyme®, 2003). In most of the clinical trials of ERT, a proportion of the patients developing antibody reactivity have developed overt reactions. In addition, some but not all of these overt reactions appear to involve complement activation.

1.6.4 Antibody effects on ERT efficacy

The presence of circulating antibodies to a replacement protein can potentially have negative effects on treatment efficacy, including altered targeting and trafficking and neutralisation of enzyme activity (Figure 1.8). Animal studies have shed some light on the effect of antibodies on ERT. In the feline model of MPS VI, significantly higher antibody titres to the replacement enzyme (rh-4-sulphatase) were reported compared to normal and untreated controls (Brooks *et al.,* 1997; Turner *et al.,* 1999). Similar titres were observed with both rh-4-sulphatase and recombinant feline-4-sulphatase, indicating that reactivity was not a species-specific response. To define the effects of circulating high titre anti-4-sulphatase antibodies, a distribution study was performed in a high and low titre rat model (Brooks *et al.,* 1998). Altered targeting, enzyme inactivation and degradation were seen in only the high titre rats. High titre rats portrayed an increased level of enzyme in the lungs and in lower density (endosomes) organelles at the subcellular level. These rats also showed a reduced level of enzyme activity one-hour post-enzyme infusion, indicating rapid removal and degradation of the replacement enzyme. Distribution changes were more evident in the high dosage-high titre rats compared to the low dosage-high or low titre rats.

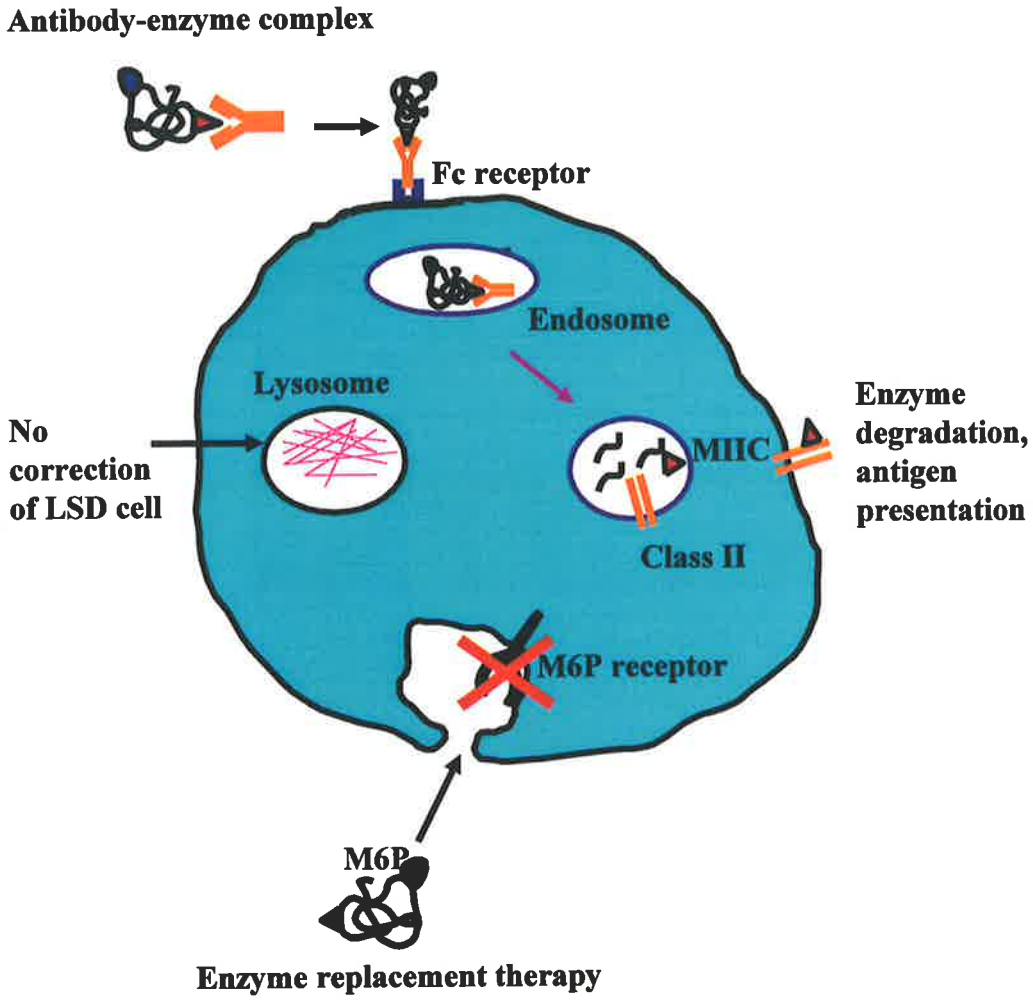

Fig. 1.8: Negative effects of antibody production on ERT efficacy

The presence of antibodies can alter the sub cellular trafficking of the replacement enzyme or lead to its degradation. Adapted from Brooks, 1999. M6P=mannose-6-phosphate.

In high and low titre rat models of MPS I a high level of antibody was required to induce changes in α-L-iduronidase targeting and distribution. Increased levels of enzyme was detected in lung, kidney and plasma compared to controls (Turner *et al.,* 2000). Altered subcellular trafficking to lysosomes was also evident with enzyme detected in endosomes; however, unlike 4-sulphatase, high titre antibodies to rh-α-L-iduronidase appeared to enhance the activity of infused protein in some rats. This suggested that the effect of immune responses may vary between individuals and that adverse effects may not always result from antibody production. Four antigenic regions were common amongst the rh-α-L-iduronidase-immunised rat sera, but the precise linear epitope sequences varied between rats. In a further study it was shown that a monoclonal antibody reacting to a single epitope near one of the four antigenic regions was capable of altering organ distribution of the infused enzyme (Glaros *et al.,* 2002).

These animal studies highlighted the fact that a high level of high affinity antibody can have dramatic effects on enzyme targeting, intracellular traffic and turnover. Although the human clinical trials suggested that altered enzyme targeting and turnover may be relatively minor, subtle decreases in the efficacy of treatment could still be mediated by antibody production and potentially alter the dose required for effective therapy.

There has been a low incidence of neutralising antibody reported in clinical trials of ERT; neutralising antibody, causing a regression in clinical improvement, has been reported in <0.5% of Gaucher disease patients (Brady *et al.,* 1997). In two Gaucher disease patients who developed an immune response to the infused enzyme it was reported that the antibodies inactivated the normal wild-type protein but not the patients' N370S mutant form of the protein (Pastores *et al.,* 1993). This is a clear example of the immune system's

ability to detect structural differences between what is considered a 'normal' and 'mutant' protein. In a recent study of antibody reactivity in enzyme-treated Fabry disease patients, 69% of patients developed neutralising sera antibodies to the α-D-galactosidase replacement protein, which coincided with continued urinary excretion of the substrate, globotriaosylceramide (Linthorst *et al.,* 2004). Evidence for the development of neutralising antibody to a replacement protein has also been reported for GSD II disease. Two of three infantile-onset GSD II patients treated with rh-α-D-glucosidase from CHO-K1 cells showed a decline in muscle function that coincided with the appearance of anti-α-D-glucosidase antibodies (Amalfitano *et al.,* 2001); in contrast, the third patient, who continued to clinically improve did not develop anti-α-D-glucosidase antibodies. Interestingly, although all GSD II patients treated with rh-α-D-glucosidase from transgenic rabbit milk developed antibody titres, it was reported that this did not relate to any adverse effect on the efficacy of the therapy (Van den Hout *et al.,* 2004; Winkel *et al.,* 2004).

1.6.5 Significance of immune response to ERT

ERT has been shown to be an effective therapy regimen for a number of LSD in both animal models and in human clinical trials. A critical question in ERT is whether an immune response is expected to arise when normal lysosomal protein is introduced to patients or corresponding animal models. Several factors may play a role in the induction of an immune response to the replacement protein, including the properties of the replacement protein (size, structure, glycosylation), the genetics of the animal/patient, the dose and route of administration, frequency of treatment, structural differences between infused and native mutant protein and the presence/absence of residual protein (Brooks, 1999). The functional enzyme deficiency in a LSD patient can result from either: no detectable protein (e.g. deletion and protein truncation mutations); a very low level of

normal protein (e.g. some mRNA splicing mutations); a low level of residual mutant protein (e.g. point mutations that alter protein conformation and/or stability); or a normal-to-high level of mutant protein (e.g. structurally conservative active site mutations) (Brooks, 1993). Therefore, the infusion of a large dose of normal lysosomal protein into a patient's circulation could be seen as foreign or at least shift the dynamic balance of enzyme in the patient (Brooks *et al.,* 2003).

It is clear from the studies in animal models and humans discussed above that some LSD patients will develop antibodies in response to ERT, which in some cases can compromise the safety of the patient and the efficacy of therapy. High titre antibody responses to ERT could potentially effect the efficacy of therapy by mediating enzyme inactivation/degradation, altered targeting and altered enzyme processing. The patient's health could also be compromised by hypersensitivity and anaphylactic reactions triggered by these high titre antibody responses. In these cases, the induction of immune tolerance becomes an important issue. A strategy for managing a Gaucher patient who had developed neutralising antibodies has been reported and includes treatment with plasma exchange, cyclophosphamide, intravenous immunoglobulin G and high-dose enzyme administration to induce immune tolerance (Brady *et al.,* 1997). A newly-developed tolerance regimen using a combination of immunosuppressive drugs was also recently described to be successful in the canine model of MPS I (Kakkis *et al.,* 2004). Although some tolerance regimens are in place, it is still important to further characterise antibody development to ERT for the management of LSD patients undergoing long-term therapy. This will consequently form the basis of this thesis.

1.7 Hypothesis and aims

ERT by intravenous infusion has been shown to be effective in treating the somatic pathology in a number of LSD and is now in clinical practice for several LSD. However, the demonstration of immune responses to ERT has indicated a potential problem for either hypersensitivity reactions during enzyme infusion or detrimental effects on the efficacy of treatment. I hypothesise that some patients will develop an immune response to ERT and that specific epitopes, which may be conserved in related enzymes, are involved in the initiation and maintenance of this antibody reactivity. Here, I define the immune reactivity of two lysosomal glycosidases, α-D-glucosidase and α-L-iduronidase. The selection of these glycosidases was in part due to the observation that both have generated immune responses in LSD patients undergoing treatment by ERT but also because they share a common catalytic mechanism and structural element (the $(\beta/\alpha)_8$ barrel). The specific aims of this thesis were:

i) investigate the progression and molecular basis of antibody responses to α-L-iduronidase;
ii) compare the antigenicity and structural epitopes between glycosidases and establish common antigenicity; and
iii) develop strategies to increase α-D-glucosidase stability and minimise its antibody reactivity

These aims will be respectively addressed in Chapters 3, 4 and 5.

CHAPTER 2:

MATERIALS AND METHODS

2 Materials and methods

2.1 Materials

Polyvinylchloride plates (96 well) were purchased from Costar (Cambridge, MA, USA) and Immulon 4 strips were purchased from Thermo Labsystems (Franklin, MA, USA). Non-protein binding plates (96 well, flat bottom) were purchased from Interpath Services (Sydney, NSW, Australia). α-D-Glucosidase and α-L-iduronidase peptide pins were synthesised by Chiron Mimotopes (Clayton, Victoria, Australia) and purchased in a 96-well format. Ovalbumin, bovine serum albumin (BSA) and 2,2 azine-di(3-ethylbenzhiazoline sulphonic acid) (ABTS) substrate were obtained from Sigma (St. Louis, MO, USA). AffiGel-10 was from Bio-Rad (Richmond, VA, USA). Peroxidase labelled sheep anti-mouse and anti-human immunoglobulin G (H + L), peroxidase labelled donkey anti-sheep/goat immunoglobulin G (H + L) and peroxidase labelled goat anti-rat immunoglobulin G (H + L) were purchased from Chemicon Australia Pty. Ltd. (Boronia, Vic, Australia). The monoclonal antibodies 43D1, 118G3 and 81D3 (against α-D-glucosidase) were a gift from Pharming BV (The Netherlands). The polyclonal antibodies to α-L-iduronidase and α-D-glucosidase were raised against purified protein (expressed in CHO-K1 cell lines; Unger *et al.*, 1994; Fuller *et al.*, 1995 or transgenic rabbit milk; Van den Hout *et al.*, 2000) and affinity purified against the respective proteins as described for another polyclonal antibody (Umapathysivam *et al.*, 2000). D-Glucose anhydrous, D-galactose anhydrous and sucrose were from Ajax chemicals (Auburn, NSW, Australia).). Culture media Coon's/Dulbecco's Modified Eagle's Medium (DMEM) was purchased from JRH Biosciences (Lenexa, Kansas, USA), Minimum Essential Medium Alpha Medium (α-MEM) was from GIBCO BRL Life technologies Inc (Grand Island, NY, USA) and Ham's F12, Basal Medium Eagle (BME) and RPMI were from ICN Biochemicals Inc. (Aurora, Ohio, USA). Foetal calf serum and trypsin-versene were purchased from JRH

Biosciences (Lenexa, Kansas, USA). Sephadex-G100 beads for gel filtration were purchased from Sigma (St. Louis, MO, USA) and pre-swollen concanavalin A-Sepharose was purchased from Amersham Pharmacia Biotech., (Uppsala, Sweden). Ultrafiltration membranes YM10 were purchased from Millipore Corporation, (Bedford, MA, USA). The fluorogenic substrates 4-methylumbelliferyl (4MU)-iduronide, 4MU-sulphate and 4MU-α-D-glucopyranoside were purchased from Sigma (St. Louis, MO, USA). The rh-α-D-glucosidase prepared from transgenic rabbit milk was a gift from Pharming BV (The Netherlands).

2.2 Tissue culture

2.2.1 Culture of CHO cells

CHO-K1 cells were maintained in 75 cm^2 tissue culture flasks containing α-MEM or Coon's/DMEM media supplemented with 10% (v/v) foetal calf serum. The cap of each culture flask was loosened prior to incubation at 37°C in 5% CO_2 and 90% humidity (Heraeus incubator) to allow gas exchange. All cell culture manipulations were conducted under sterile conditions in a biohazard hood within a cell culture facility.

2.2.1.1 rh-α-D-Glucosidase expression with and without additional D-glucose

For glucose experiments, the culture medium was exchanged for serum-free α-MEM or Coon's DMEM supplemented with sterile D-glucose at 1.0, 3.1, 4.0 and 6.0 g/L final concentration. Media samples (100 μL) were taken at 0, 4, 24, 54, 72, 98 and 144 hours after the addition of D-glucose to determine the level of α-D-glucosidase activity using a 4MU substrate (section 2.7.1) and protein analysis using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; section 2.9).

For α-D-glucosidase purification, ten 75 cm^2 tissue culture flasks of CHO-K1 cells over-expressing rh-α-D-glucosidase (Fuller *et al.,* 1995) were grown to confluency, harvested and transferred to a 175 cm^2 factory (Nunc™ Serving Life Science, Denmark) using a sterile 60 cc/mL syringe (Terumo® Syringe Leur lock tip). Cells were grown in 200 mL of Coon's/DMEM medium supplemented with 10% (v/v) foetal calf serum and 50 μg/mL streptomycin. At confluency, cells were incubated with 200 mL of serum-free medium supplemented with D-glucose at a final concentration of 6 g/L (expression media) for four days. Medium was collected, clarified by centrifugation at 400 x g for 5 min and stored at 4°C ready for purification. The factory was continuously maintained, using the expression media, until cell senescence was observed.

2.2.1.2 Expression of rh-α-D-glucosidase, rh-α-L-iduronidase and rh-4-sulphatase

To evaluate the effect of different sugars on rh-α-D-glucosidase, rh-α-L-iduronidase and rh-4-sulphatase expression, an activity time course was conducted. CHO-K1 cells over-expressing rh-α-D-glucosidase, rh-α-L-iduronidase and rh-4-sulphatase (Fuller *et al.,* 1995, Unger *et al.,* 1994, Anson *et al.,* 1992 respectively) were cultured as in section 2.2.1 and at confluency, media was exchanged for serum-free Coon's/DMEM containing no added sugar, or 6.0 g/L of either D-glucose, sucrose, D-galactose or a non-sugar energy source, 0.1 M butyric acid. Media samples (250 μL) were collected at 0, 5, 24, 48, 72, 96, 120, 144 and 168 hours post-addition and evaluated for α-D-glucosidase, α-L-iduronidase and 4-sulphatase activity using 4MU substrates (sections 2.7.1-2.7.3).

2.2.2 Culture of GSD II patient skin fibroblasts in the presence of D-glucose

GSD II patient skin fibroblasts, including six infantile-onset, four adult-onset and a normal cell line were revived from cryopreservation. Cell lines were cultured in BME

supplemented with 10% (v/v) foetal calf serum. Upon confluency, cells were washed with sterile Phosphate-buffered salin (PBS) and incubated with fresh BME containing 10% foetal calf serum and either 6, 25 or 50 mg/mL of sterile D-glucose. Cells were incubated for three-days then harvested (section 2.2.3) and cell lysates prepared by sonication (section 2.2.4). Cell lysates were evaluated for total protein (section 2.8.2), α-D-glucosidase activity (section 2.7.1) and α-D-glucosidase protein by the multiplex immune quantification method (section 2.3.5).

2.2.3 Cell harvesting

Cultured CHO-K1 and skin fibroblast cells were harvested with 10% (v/v) trypsin/versene in phosphate-buffered saline (PBS; 5 mL) at room temperature by incubation for one minute. Trypsinised cells were transferred to a new 75 cm^2 tissue culture flask containing 10 mL of growth medium supplemented with 10% (v/v) foetal calf serum. CHO-K1 cells were subcultured 1:10 twice per week and skin fibroblasts were subcultured 1:2 weekly.

2.2.4 Cell lysate preparation

CHO-K1 cells were harvested as described in section 2.2.3, centrifuged at 400 x g for 5 min and cell pellets resuspended in 100 µL of lysis solution (0.02 M Tris(hydroxymethyl)aminomethan-Hydrocloric acid (Tris-HCl), pH 7, containing 0.5 M Sodium chloride (NaCl)). Cell lysates were prepared by six-cycles of freeze-thawing. Alternatively, the resuspended pellets were sonicated on ice for 20 seconds using the Ystrom sonicator (Systems U.S.A.). The cell suspension was clarified by centrifugation at 11,000 x g in the microcentrifuge (Biofuse fresco, Heraeus) for 5 min at 4°C. The supernatant was removed and stored for analysis, and the pellet containing the cell debris

discarded. Cell lysates were assayed for total protein by Bianchoninic acid (BCA) method (section 2.8.1) or Lowry (section 2.8.2) and then analysed by SDS-PAGE (section 2.9).

2.2.5 Culture of hybridoma cell lines

Hybridomas expressing monoclonal antibodies against α-D-glucosidase (43D1, 118G3, 81D3, 81A6, 43G8) were revived from cryopreservation and cultured in RPMI media supplemented with 10% (v/v) foetal calf serum. Culture media containing antibody was collected after 3-to 4-days of culture and clarified by centrifugation at 400 x g for 5 min and the supernatant stored at -20°C.

2.2.6 Cryopreservation of hybridoma, fibroblast and CHO-K1 cells

A cell count was carried out to ensure greater than 80% cell viability for cryopreservation. Cells were centrifuged at 400 x g for 5 min. Cells were resuspended in 50% (v/v) heat-inactivated foetal calf serum and 50% (v/v) cell culture medium to half the final volume required to obtain a concentration of 6-10 x 10^6 cells/mL. To make up the final volume a solution of 30% dimethyl sulphoxide (DMSO; Sigma) in cell culture medium was added drop-wise, with continuous mixing. An aliquot of 1.5 mL was transferred to a 1.8 mL polypropylene cryopreservation tube (CryoTube™ vials, NUNC™ Serving Life Sciences, Denmark) and placed in a cryo-freezing container at -80°C for 12 hours. The frozen cells were then transferred to liquid nitrogen.

2.2.7 Thawing of cryopreserved hybridoma, skin fibroblast or CHO-K1 cells

A vial of frozen cells was thawed at 37°C and transferred to a 30 mL V bottom tube with a Pasteur pipette. An equal volume of media was added to the cells drop-wise, with continuous mixing, and the cells were then incubated at room temperature for 10 min. A

further 10 mL of medium was added and the cells centrifuged at 400 x g for 5 min. Cells were resuspended in 20 mL of medium and centrifuged at 400 x g for a further 5 min. Cells were then resuspended in 5 mL of medium, transferred to a 25 cm^2 tissue culture flask and maintained in a 37°C incubator under 5% CO_2. Once confluent, cells were harvested and transferred to a 75 cm^2 tissue culture flask.

2.3 Immune assays

2.3.1 Evaluation of sera samples for antibody titre

Sera antibody titres to α-L-iduronidase were determined by an ELISA. A 50 µL aliquot of rh-α-L-iduronidase, at a concentration of 10 µg/mL in 0.1 M Sodium hydrogen carbonate ($NaHCO_3$), pH 8.5, was added to each well of a 96-well polyvinyl chloride plate and incubated overnight at 4°C. Unbound antigen was aspirated from each well and the wells then washed three times with 0.02 M Tris-HCl, pH 7, containing 0.025 M NaCl, using a microtitration plate washer (ADIL Instruments, France). The remaining reactive sites on the plate were blocked by the addition of 200 µL of 1% (w/v) ovalbumin in 0.02 M Tris-HCl, pH 7, containing 0.25 M NaCl, for 3 hours at 20°C. Test and control sera were albumin-absorbed with ovalbumin/ BSA coupled to Affi-Gel 10, by overnight incubation at 4°C with rotation. Sera were serially diluted in 1% (w/v) ovalbumin in 0.02 M Tris-HCl, pH 7, containing 0.25 M NaCl, and 50 µL was added to each well overnight at 4°C. Unbound sera was aspirated with three washes of 0.02 M Tris-HCl, pH 7, containing 0.25 M NaCl. Wells were then incubated with 100 µL of peroxidase-conjugated sheep anti-human immunoglobulin (Silenus, Hercules, CA, USA) at a 1/1000 dilution in 0.02 M Tris-HCl, pH 7, containing 0.25 M NaCl, for 1 hour at 20°C. Unbound antibody was aspirated and wells washed three times with 0.02 M Tris-HCl, pH 7, containing 0.25 M NaCl. A 100 µl aliquot of ABTS substrate (Sigma, Aldrich) was then added to each well for 20 min at

20°C for colour development. The optical density units (OD) of each well was measured at 410 nm on an automated ELISA plate reader (Ceres 900 Hdi, Bio Tek Instruments Inc., USA). Results were compared to a non-sera negative control (1% (w/v) ovalbumin in 0.02 M Tris-HCl, pH 7, containing 0.25 M NaCl), to correct for background reactivity. Antibody titre was determined as the lowest dilution of sera sample at which the OD at 410 nm was greater than two standard deviations above background.

Sera from mice and rats infused with rh-α-D-glucosidase were evaluated for antibody titres to rh-α-D-glucosidase as described above, with the following alterations. Wells were coated with rh-α-D-glucosidase purified from either transgenic rabbit milk or CHO-K1 cells. Different detection antibodies were used including either peroxidase-conjugated sheep anti-mouse immunoglobulin (Silenus, Hercules, CA, USA) or peroxidase-conjugated goat anti-rat immunoglobulin (Southern Biotechnology Associates Inc., USA)).

Monoclonal and polyclonal antibody reactivity against rh-α-D-glucosidase or rh-α-L-iduronidase was evaluated by an ELISA as described above, with appropriate detection antibodies (peroxidase-conjugated sheep anti-mouse immunoglobulin or peroxidase-conjugated donkey anti-sheep immunoglobulin). For antibody reactivity against denatured enzyme forms, a microtitre plate was coated with enzyme that had been either boiled (semi-denatured form) and/or boiled with 5% (v/v) β-mercaptoethanol (denatured form).

2.3.2 Epitope mapping

Peptide pin technology (Chiron Mimotopes, Clayton, Vic, Australia) was used to determine the linear sequence epitope reactivity of enzyme-treated MPS I patient sera antibodies, as previously described for another lysosomal protein (Turner *et al.,* 1999).

Briefly, individual 13 amino acid peptides spanning the α-L-iduronidase protein were synthesised onto polyethylene pins (Geysen *et al.,* 1984). An overlap of six amino acids between consecutive peptides was used to avoid splitting of linear sequence epitopes. The array of peptide pins (in a 96-well format) was subjected to an ELISA (see section 2.3.1) to quantify linear sequence epitope reactivity. In defining antibody epitopes on α-L-iduronidase, it was assumed that there was a single antibody interaction per 12-mer peptide. The level of ELISA reactivity with each individual peptide was therefore a crude measure of antibody affinity. Absorbances of 0 to 0.699 OD units were defined as 'little' or 'no reactivity', absorbances of 0.7 to 2.499 OD units were defined as 'low affinity reactivity', and OD units greater than 2.5 units were defined as 'high affinity reactivity'. As a quality control all results were compared to positive and negative control pins. Similarly, linear sequence epitope reactivity of sera antibodies from rh-α-D-glucosidase-infused mice and rats, and of anti-α-D-glucosidase monoclonal and polyclonal antibodies, were evaluated as described above, but on peptide pin plates of α-D-glucosidase and with appropriate detection antibodies (peroxidase-conjugated sheep anti-mouse immunoglobulin, and peroxidase-conjugated goat anti-rat immunoglobulin). Linear sequence epitope cross-reactivity of antibodies raised against α-D-glucosidase and α-L-iduronidase were respectively evaluated on peptide pin plates of α-L-iduronidase and α-D-glucosidase as described above. Peptide pin plates were cleaned and a conjugate test performed to determine background reactivity as described by the manufacturer (MULTIPIN™ Synthesis Kit Software Manual, Chiron Mimotopes, Australia)

2.3.3 Temperature denaturation of rh-α-D-glucosidase

Two forms of rh-α-D-glucosidase were obtained from a rh-α-D-glucosidase, CHO-K1 expression cell line (Fuller *et al.,* 1995); a precursor form (secreted) and a proteolytically

processed form (intracellular, from cell lysates). The effect of temperature treatment on the conformation of precursor and proteolytically processed rh-α-D-glucosidase was evaluated by ELISA with monoclonal antibodies. A 50 µl aliquot of affinity purified anti-α-D-glucosidase polyclonal antibody at a concentration of 10 µg/ml in 0.1 M $NaHCO_3$, pH 8.5, was added to each well of a polyvinylchloride plate and incubated overnight at 4°C. Unbound polyclonal antibody was aspirated and wells were washed three times with 0.02 M Tris-HCl, 0.25 M NaCl, pH 7, using a microplate plate washer (ADIL instruments). Any remaining reactive sites on the wells were blocked with 200 µl of 1 % (w/v) ovalbumin in 0.02 M Tris-HCl, 0.25 M NaCl, pH 7 (blocking solution), for 5 h at room temperature. Plates were then washed three times with 0.02 M Tris-HCl, 0.25 M NaCl, pH 7. Precursor and proteolytically processed forms of rh-α-D-glucosidase, at a concentration of 0.065 µg/ml in blocking solution, were treated at temperatures of 25, 50, 52.5, 55, 57.5, 60, 62.5, 65, 67.5, 70, 72.5, and 75°C. Fifty microlitre aliquots of each heat-treated sample was added to separate ELISA wells and incubated overnight at 4°C. Unbound α-D-glucosidase protein was then aspirated and the plates washed three times with 0.02 M Tris-HCl, 0.25 M NaCl, pH 7. Anti-α-D-glucosidase monoclonal antibodies (culture supernatant, 100 µl) were added to the ELISA plate wells and incubated at room temperature for 5 h. Unbound monoclonal antibody was then aspirated and ELISA plates washed for a further three times. Each well was then incubated with 100 µl of peroxidase-conjugated sheep anti-mouse immunoglobulin at a 1/100 dilution in 0.02 M Tris-HCl, 0.25 M NaCl, pH 7, for 1 h at room temperature. Unbound antibody was then removed and the plates washed for a further three times. ABTS substrate (100 µl) was then added to each well and incubated at room temperature for 20 min. The absorbance of each well was subsequently measured at 405 nm on an automated ELISA plate reader (BIO-Tek Ceres 900).

2.3.4 Immune quantification assay for lysosomal proteins

A two-step time-delayed dissociation-enhanced lanthanide fluorescence immune-assay (DELFIA) was utilised to evaluate the inhibition effect of D-glucose on rh-α-D-glucosidase, rh-α-L-iduronidase and rh-4-sulphatase, antibody reactivity. Briefly, microtitre plates (Immulon 4, Dynatech Laboratories, Inc.) were coated with capture antibody (either sheep anti-α-D-glucosidase polyclonal antibody, sheep anti-α-L-iduronidase polyclonal antibody or anti-4-sulphatase monoclonal antibody) at a concentration of 5 μg/mL in 0.1M $NaHCO_3$ (100 μL/well) and incubated overnight at 4°C. Plates were then washed (3X) with DELFIA wash buffer (0.02 M Tris-HCl, pH 7, containing 0.25 M NaCl, 0.005% (v/v) Tween 40 (BDH, Poole, England), 0.002% (w/v) Thiomerosal, pH 7.8) in the DELFIA plate washer (Wallac, Turku, Finland) and blocked with 200 μL/well of 0.02 M Tris-HCl, pH 7, containing 0.025 M NaCl and 1% (w/v) ovalbumin, for 1 hour at room temperature. Microtitre plates were washed with DELFIA wash buffer (2X) and then 50 μL of protein standards (either α-D-glucosidase, α-L-iduronidase or 4-sulphatase protein standards) diluted in DELFIA assay buffer (0.05 M Tris-HCl, 0.15 M NaCl, 20 μM diethylenetriamine-penta-acetic acid, pH 7.8, containing 0.01% (v/v) Tween 40, 0.5% (w/v) BSA, 0.05% (w/v) bovine gamma-globulin, and 0.05% (w/v) sodium azide) were added to separate wells. Test samples (rh-α-D-glucosidase purified from CHO cells or from transgenic rabbit milk (Pharming), rh-α-L-iduronidase protein, rh-4-sulphatase protein) were diluted in DELFIA assay buffer to concentrations of 40, 20, 10, 5, 2.5 and 1.25 ng/mL and 50 μL added to separate wells. DELFIA assay buffer containing 0, 6 or 50 mg/mL of D-glucose (50 μL/well) was added to separate wells. The plates were covered and incubated for 1 hour at room temperature, with shaking, on the DPC Milenia Micromix 4 Plate Shaker (form:99, amplitude 5) followed by a 16 hour incubation at 4°C. The plate was equilibrated to room temperature for 1 hour and

microtitre wells were washed twice with DELFIA wash buffer. The europium-labelled detection antibodies (monoclonal anti-α-D-glucosidase antibody (43D1), monoclonal anti-α-L-iduronidase antibody (ID1A), polyclonal anti-4-sulphatase antibody) were diluted to 400 ng/mL in assay buffer containing 0, 6 or 50 mg/mL D-glucose and 50 µl added to corresponding wells. The plate was covered and incubated with shaking for 1 hour at room temperature. After washing six-times with DELFIA wash buffer, 200 µL of DELFIA® enhancement solution (Wallac, Turku, Finland) was added to each well. Wells were agitated for 10 min at room temperature before measuring fluorescence on the DELFIA® 1234 Research Fluorometer (Wallac, Turku, Finland).

2.3.5 Multiplex analysis of lysosomal proteins

Multiplexing is based upon the immunological measurement of multiple analytes from a single sample on the Bio-Plex array system (Bio-Rad, USA) that utilises flow cytometry xMap technology (Luminex Corporation). The multiplex was utilised to analyse α-D-glucosidase protein levels in GSD II patient skin fibroblast cell lysates that had been treated with D-glucose. Wells of a 96-well microtitre filter plate were pre-wet with 100 µL of assay buffer (0.05% (v/v) Tween 20, 0.5% (w/v) BSA, 0.05% (w/v) gammaglobulin in 0.01 M Sodium dihydrogen orthophosphate (NaH_2PO_4)/ Sodium hydroxide (NaOH) pH 7.4 with 150 mM NaCl plus 0.05% (w/v) sodium azide) and the buffer was then suctioned off before the addition of 50 µL α-D-glucosidase polyclonal antibody-coupled beads (5000 beads, coupled according to the manufacturer's instructions; BioRad, USA), to each well. The buffer was suctioned off and then either α-D-glucosidase standards serially diluted in assay buffer were added (0-1000 pg in 100 µL, assayed in duplicate) or 100 µL of assay buffer added as blanks or GSD II patient skin fibroblast cell lysates (2 µL in 100 µL of assay buffer) added to the microtitre wells. The α-D-glucosidase reporter antibody

(biotinylated: Biotinylation of antibodies was performed with a FluoReporter®Biotin-XX Labeling Kit according to the manufacturer's instructions; Molecular Probes Inc. Eugene, OR, USA) was diluted in assay buffer (320 μg/L) and 50 μL added to each well. The plates were then covered in foil and shaken at room temperature (150 rpm for 1 h on the Thermoline Orbital Shaker; Thermoline Scientific Equipment Pty. Ltd, Australia) and then incubated overnight at 4°C. Wells were allowed to reach room temperature by shaking at 150 rpm for 1 hour and then washed three times with 100 μL of wash buffer (0.01 M NaH_2PO_4/NaOH pH 7.4 with 150 mM NaCl, containing 0.05% (w/v) Tween 20). The buffer was suctioned off and 125 μL of streptavidin phycoerythrin (1.2 mg/L in assay buffer) was added at 50 ng/well (400 μg/L), then the plate sealed and shaken at 150 rpm for 10 minutes at room temperature. The plate was uncovered and fluorescence measured on a Bio-Plex array system (Bio-Rad, USA). All results were interpolated through a standard curve to calculate the concentration of α-D-glucosidase.

2.4 Infusion of rh-α-D-glucosidase into animal models

2.4.1 rh-α-D-Glucosidase immunised rats

Six-week old female Sprague Dawley rats (n=8) were subcutaneously injected fortnightly for eight-weeks with rh-α-D-glucosidase (100 μg/injection) derived from either transgenic rabbit milk (Pharming BV, The Netherlands) or from CHO-K1 cells. The primary immunisation consisted of rh-α-D-glucosidase with Freund's complete adjuvant at a 1:1 ratio and the subsequent three boosters with incomplete Freund's adjuvant. An emulsion of the rh-α-D-glucosidase and adjuvant was prepared and injected subcutaneously. Forty-eight hours post the final immunisation 5-10 mL of blood was collected by heart puncture

(2.5.1). Non-immunised rats (n=4) acted as controls for antibody reactivity against rh-α-D-glucosidase.

2.4.2 Subcutaneous infusion of rh-α-D-glucosidase in mice

Female C57Bl/6 mice at six- to eight-weeks of age (n=10) were subcutaneously administered 1 mg/kg of rh-α-D-glucosidase purified from either transgenic rabbit milk (Pharming BV, The Netherlands) or CHO-K1 cells. rh-α-D-Glucosidase was administered fortnightly over an eight-week duration to assess antibody titre production. Blood samples (1% of body weight) were collected fortnightly from the tail vein as described in section 2.5.2. Forty-eight hours after the final infusion, blood (2 mL) was collected by heart puncture (2.5.1). Non-infused mice (n=5) acted as controls for antibody reactivity against α-D-glucosidase

2.5 Blood collection and sera sample preparation

2.5.1 Heart puncture

Blood collection by heart puncture was conducted under sterile conditions in a laminar flow hood. The rats/mice were anaesthetised using 4% (v/v) isofluorane and 1.5-2 L O_2 in an anaesthetising box. Anaesthetised animals were then transferred to a working area within the laminar flow hood. An anaesthetic mask was placed over the mouth of each animal and the foot-pad pinch test was utilised to determine complete anaesthesia. A 21G needle was inserted into the heart; the beating movement of the needle and the presence of blood in the needle hub indicated the correct positioning of the needle. The syringe was then attached to the needle and blood drawn. Blood was placed into a container and left to stand at room temperature for 30 min for blood clot formation and then incubated at 4°C overnight. Using the same needle in the heart, 0.5-1 mL of lethabarb was injected for

euthanasia. Blood clots were dislodged from the container and centrifuged at 700 x g for 5 min. Supernatant was collected and re-centrifuged to sediment any debris collected. Supernatant (sera) was collected, pre-cleared by albumin absorption (section 2.5.4) and stored at –20°C for antibody titre (section 2.3.1) and epitope mapping (section 2.3.2) experiments.

2.5.2 Tail vein blood sampling from rats and mice

Blood collection from the tail vein of rats and mice was conducted fortnightly throughout the immunisation/infusion period. Animals were restrained and the tail heated using a heating lamp to visualise the lateral tail veins. Once visible, a needle (27G for mice, 25G for rats) was inserted into the tail vein and blood allowed to fill into the needle hub. Blood was collected by capillary action using a glass Pasteur pipette; 1% of total body weight of blood was collected into a centrifuge tube and placed on ice at 4°C prior to centrifugation at 11 000 x g for 10 min. Supernatant containing the sera antibodies was collected, pre-cleared by albumin absorption (section 2.5.4) and stored at -20°C for antibody titre determination.

2.5.3 Preparation of ovalbumin/BSA-bound Affi-Gel 10

Affi-Gel was used to couple proteins by amide linkages. Protein to be coupled (1% (w/v) ovalbumin and 1% (w/v) BSA) was dissolved in 0.1 M sodium bicarbonate, pH 8.5. Affi-Gel 10 (Bio-Rad, USA) was washed with two volumes of ice-cold water for injection (4X) by a 30 secs 400 x g spin. The Affi-Gel 10 was mixed with an equal volume of protein solution in a tube sealed with parafilm and incubated on a rotator at 4°C overnight. Excess protein was removed and the protein-coupled Affi-Gel was washed (4X) with 0.02 M Tris-HCl, pH 7, containing 0.25 M NaCl to block excess amine groups. Protein coupled Affi-

Gel was mixed in a tube with an equal volume of 0.02 M Tris-HCl, pH 7, containing 0.25 M NaCl, sealed with parafilm and stored at 4°C.

2.5.4 Albumin absorption of sera samples

Sera samples were albumin-absorbed with an excess of ovalbumin/BSA-coupled Affi-Gel 10 overnight at 4°C. The Affi-Gel-sera slurry was centrifuged at 11,000 x g in a microcentrifuge at 4°C for 5 min to sediment the Affi-Gel. Supernatant containing the sera antibodies was collected and stored at -20°C for antibody reactivity evaluations.

2.6 rh-α-D-Glucosidase purification from CHO-K1 expression cells

2.6.1 rh-α-D-Glucosidase purification

Purification of precursor rh-α-D-glucosidase was conducted as previously described for rh-α-D-glucosidase purification from human urine (Oude Elferink *et al.,* 1984) and adapted for CHO-K1 culture medium (Fuller *et al.,* 1995). Briefly, the medium from a CHO-K1 cell factory expressing rh-α-D-glucosidase (section 2.2.1.1) was clarified by centrifugation at 400 x g (Mistral 3000i centrifuge) for 5 min at 4°C to remove cells and debris. The pH was adjusted to pH 6.6 to preserve enzyme activity, loaded (1 L) onto a 10 mL Concanavalin A-Sepharose (Pharmacia Biotech) column and run under gravity at 4°C. The column was then washed with three column volumes of 10 mM sodium phosphate, pH 6.6, containing 100 mM NaCl. This was followed with three washes of the same buffer containing a final concentration of 1 M NaCl and then with one column volume of wash buffer containing a final concentration of 0.5 M NaCl. The column was eluted with five column volumes of the latter buffer containing 1 M methyl-glucoside and 5 mL fractions were collected. Eluted fractions were assayed for α-D-glucosidase activity (section 2.7.1)

and active fractions were pooled and concentrated by Amicon ultrafiltration using a YM10 Diaflo membrane. In the ultrafiltration unit, the eluate was dialysed against 20 mM sodium acetate, pH 5.2, containing 25 mM NaCl. The concentrated glycoprotein fraction (4 mL) was loaded onto a Sephadex G-100 (Sigma Aldrich) column (50 cm X 1.5 cm) and fractioned in the same dialysis buffer at a rate of 20 min/hour. Fractions of 2 mL were collected and assayed for α-D-glucosidase activity, and pooled and concentrated using the Amicon ultrafiltration unit. Concentrated eluate was assayed for α-D-glucosidase activity (section 2.7.1) and also for total protein by BCA (section 2.8.1). Enzyme for infusion was dialysed against 0.01 M PBS, pH 7.2, using Membra-CEL dialysis tubing (10 kDa molecular weight cut-off) and filtered through a MILLEX-GV® 0.22 μm sterile filter unit.

2.6.2 Modified rh-α-D-glucosidase purification

rh-α-D-Glucosidase was purified as described in section 2.6.1, with the following modifications. An equal volume of 50 mM sodium acetate and 20 mM sodium phosphate, 1 M NaCl, pH 5.2 (as described in Van Hove *et al.,* 1997) was added to clarified tissue culture medium to stabilise α-D-glucosidase activity. This was loaded (2 L) onto a 10 mL Concanavalin A-Sepharose column and allowed to run under gravity at 4°C. The column was washed with 50 mL of wash buffer (50 mM sodium acetate, 20 mM NaH_2PO_4, 1 M NaCl, pH 5.2) and eluted with 15 mL of the same buffer containing 1 M methyl-glucoside. Elution was maximised by passing the eluate over the column three-times followed by a further 5 mL elution with fresh elution buffer. Prior to concentration, 125 mg/mL of D-glucose and 0.1% (v/v) of Tween 20 (BDH, Poole, England) was added to the Concanavalin-A eluate and the YM10 membrane soaked in detergent solution (0.1% (v/v) Tween 20 in distilled water). The concentrated eluate was applied to the Sephadex G-100 column as previously described (2.6.1) using 20 mM sodium acetate, pH 4.6, containing 25

mM NaCl running buffer. Fractions were assayed for α-D-glucosidase activity; active fractions were pooled and concentrated five-fold in an Amicon ultrafiltration unit using an YM10 membrane that had been pre-soaked in detergent solution. The concentrated eluate was dialysed against PBS and filtered through a MILLEX-GV® 0.22 μm sterile filter unit that had been pre-soaked in detergent solution.

2.7 Enzyme activity assays

2.7.1 α-D-Glucosidase activity

α-D-Glucosidase activity was measured by a fluorogenic assay using the artificial substrate 4MU-α-D-glucopyranoside, as described by Fugimoto and Fluharty (1978). Briefly, test and control samples were diluted in 0.9% (w/v) saline. In plastic serology tubes, 50 μL of diluted sera was incubated with 50 μL of the fluorogenic substrate (3.25 mM in 0.1 M sodium acetate buffer, pH 4) at 37°C for 60 min. The reaction was stopped with the addition of 1.6 mL stop buffer (0.2 M glycine, pH 10.7 containing 0.157 M Sodium carbonate (Na_2CO_3), 0.125 M NaOH). Duplicate 20 μL aliquots of 4-methylumbelliferone (4MU) standard (0.142 M) in 1.6 mL 0.2 M glycine, pH 10.7, containing 0.157 M Na_2CO_3, 0.125 M NaOH, were prepared. Relative fluorescence of each tube was measured on the spectrophotofluorimeter (Perkin Elmer LS-5) using an excitation and emission wavelength of 366 and 446 nm. One relative fluorescence unit (RFU) of activity was defined as the amount of enzyme required to produce 1 nanamole of 4MU per min.

2.7.2 α-L-Iduronidase activity

α-L-Iduronidase activity was assayed by a fluorogenic assay using the artificial substrate 4MU-iduronide as described in Clements *et al.,* (1985). Test and control samples were

diluted 1 in 10 in dilution buffer (0.35% (w/v) BSA in 0.1 M dimethylglutarate buffer, pH 4.5). In plastic serology tubes, 5 μL of diluted sample was incubated with 10 μL of fluorogenic substrate (30% (w/v) of 4MU-iduronide in 0.1 M dimethylglutarate buffer, pH 4.5) at 37°C for 20 min. The reaction was terminated with the addition of 1.6 mL of 0.2 M glycine, pH 10.7, containing 0.157 M Na_2CO_3, 0.125 M NaOH. Duplicate 20 μL aliquots of 4MU standard (0.142 M) in 1.6 mL 0.2 M glycine, pH 10.7, containing 0.157 M Na_2CO_3, 0.125 M NaOH, were prepared. Relative fluorescence of each tube was measured on the spectrophotofluorimeter (Perkin Elmer LS-5) using an excitation and emission wavelength of 366 and 446 nm.

2.7.3 4-Sulphatase activity

4-Sulphatase activity was assayed using the fluorogenic substrate 4MU-sulphate (Gibson *et al.,* 1987). In plastic serology tubes, 90 μL of assay mix (5 mM 4-MU-Sulphate, 50 mM sodium acetate, pH 5.6, and 0.05 mg/mL BSA) was incubated with 10 μL of diluted test sample (50 mM sodium acetate, pH 5.6, containing 0.05% (w/v) Tween 20) at 37°C for 20 min. The reaction was terminated by the addition of 1.6 mL of glycine stop buffer (0.2 M glycine, pH 10.7, containing 0.157 M Na_2CO_3, 0.125 M NaOH). Duplicate 20 μL aliquots of 4MU standard (0.142 M) in 1.6 mL 0.2 M glycine buffer, pH 10.7, were prepared. Relative fluorescence of each tube was measured on the spectrophotofluorimeter (Perkin Elmer LS-5) using an excitation and emission wavelength of 366 and 446 nm.

2.7.4 Enzyme inhibition studies

Kinetic experiments with the fluorogenic substrates used 0 to 5 mM of either 4MU-α-D-glucoside or 4-MU-iduronide, in the presence of either 0, 6, 25 or 50 g/L D-glucose (0, 33, 139, 278 mM). Lineweaver-Burk plots for both α-D-glucosidase and α-L-iduronidase were

used to determine the Michaelis menten constant (K_m) and maximum velocity of catalysis (V_{max}) for each condition. The inhibitory constant (K_i) value was calculated from plots of slope (from the Lineweaver-Burk plots) versus D-glucose inhibitor concentration.

2.8 Protein estimation

2.8.1 BCA method for protein determination

The Micro BCA™ Protein Assay Reagent Kit (PIERCE) was utilised. BSA standards ranging from 0 to 20 µg were prepared in Milli Q water and pipetted (duplicates) into wells (100 µL) of a non-protein binding 96-well plate (Greiner Bio-One, Germany). Test samples were diluted 20X with Milli Q water and 100 µL added to wells. The micro BCA™ Working Reagent was prepared and 100 µL/well added to standards and test samples. The plate was covered, incubated at 37°C for 2 hours and the absorbance read on the ELISA microplate reader (Ceres 900 Hdi, Bio Tek Instruments Inc., USA) at 540-590 nm. The protein concentration of unknown samples was estimated from a BSA standard curve.

2.8.2 Lowry method for protein determination

Total protein estimation by the Lowry method was conducted as previously described (Lowry *et al.,* 1951). Briefly, protein standards (Protein Standard Solution, Sigma, USA) were prepared in plastic serology tubes ranging from 32 µg/100 µL to 4 µg/100 µL in 0.9% (w/v) saline. Test samples were diluted 20X in 0.9% (w/v) saline to a final volume of 100 µl in plastic serology tubes. The following reagents were prepared: reagent A (2% (w/v) sodium carbonate and 0.4% sodium hydroxide in deionised water); reagent B (1.7% (w/v) trisodium citrate dihydrate in distilled water); reagent C (0.1% (w/v) cupric sulphate in distilled water); and reagent D (1% (v/v) reagent B, 0.1% (v/v) reagent C in 10 mL of

reagent A). Reagent D (1.0 mL) was added to each tube, vortexed and incubated at room temperature for at least 1 minute. Diluted Folin-Ciocoulteau's Reagent (Merck) was diluted at a 1:1.5 ratio in distilled water, 100 μL was then added to each tube and vortexed immediately after addition. Tubes were incubated for 30 min at room temperature before reading the absorbance on a spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences, Australia) at a wavelength of 750 nm against a reagent blank.

2.9 Western blot analysis

Samples were denatured for SDS-PAGE by boiling at 100°C for 10 min with 4X sample buffer (0.4% (w/v) Bromophenol blue, 40% (v/v) glycerol, 8% (w/v) sodium dodecyl sulphate in 200 mM Tris, pH 6.8) and 5% (v/v) β-mercaptoethanol. Samples were separated on a 10% resolving gel using standard conditions (Laemmli, 1970) and then transferred onto a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech) at 500 mA for 1 hour. After transfer, the non-specific protein binding sites on the nitrocellulose membrane were blocked by incubating with 5% (w/v) BSA in 0.02 M Tris-HCl, pH 7, containing 0.25 M NaCl, for 1 hour at room temperature, with rocking. The membrane was washed three-times with 0.02 M Tris-HCl, pH 7, containing 0.25 M NaCl. For detection of α-D-glucosidase antigen, affinity-purified polyclonal anti-α-D-glucosidase antibody was diluted to 1 μg/mL in blocking solution and 10 mL added to the membrane overnight at 4°C, with rocking. The nitrocellulose was washed three-times, as above, and incubated with 10 mL of a horseradish peroxidase–conjugated donkey-anti-sheep immunoglobulin diluted 1/1000 in blocking solution for 1 hour at room temperature. The blot was washed and developed with the 4-chloro-1-napthol substrate (Sigma; 60 mg dissolved in 20 mL ice-cold methanol and then combined with 100 mL 0.02 M Tris-HCl, pH 7, containing 0.25 M NaCl and 60 μL hydrogen peroxide). Development was

terminated by rinsing the membrane with several changes of water. Band sizes were compared to the BenchMark™ Prestained Protein Ladder (Invitrogen Life Technologies, California).

CHAPTER 3:

IMMUNE REACTIVITY IN MPS I

3 Introduction

ERT was recently approved for clinical use in MPS I patients, but 91% of patients so far tested were reported to develop antibodies to rh-α-L-iduronidase (Aldurazyme®, 2003). This study aimed to investigate the progression and molecular basis of antibody responses to rh-α-L-iduronidase in mice and humans receiving ERT. Antibody titres were determined in ERT-treated α-L-iduronidase knockout mice and in MPS I patients involved in a phase I/II clinical trial. The linear sequence epitope reactivity of antibodies produced in response to ERT in MPS I patients was defined and the structural nature of the immune response to the rh-α-L-iduronidase characterised. The investigation of antibodies produced in response to ERT has direct relevance to the safety and efficacy of LSD patient treatment.

3.1 Results

3.1.1 Antibody response in rh-α-L-iduronidase-treated mice

A panel of sera samples from enzyme-treated and control mice were obtained from Lorne A. Clarke (Department of Medical Genetics, British Columbia Research Institute for Children's and Women's Health, University of British Columbia, Vancouver, Canada). The mouse phenotype and administered treatment were blinded until antibody titres to the α-L-iduronidase replacement enzyme were evaluated. The sera antibody titre levels to rh-α-L-iduronidase in the various treatment groups were defined (Table 3.1). Normal mice and iduronidase-deficient untreated mice developed low-level antibody titres (400-3,200). This was consistent with the background antibody titres seen in other animal models and in humans prior to the onset of therapy (Brooks *et al.*, 1997; Daly *et al.*, 2000; Kakkis *et al.*, 2001; Linthorst *et al.*, 2004). Recombinase-activating gene (Rag)-1-deficient mice lack functional lymphocytes because of an inability to properly rearrange antigen receptors,

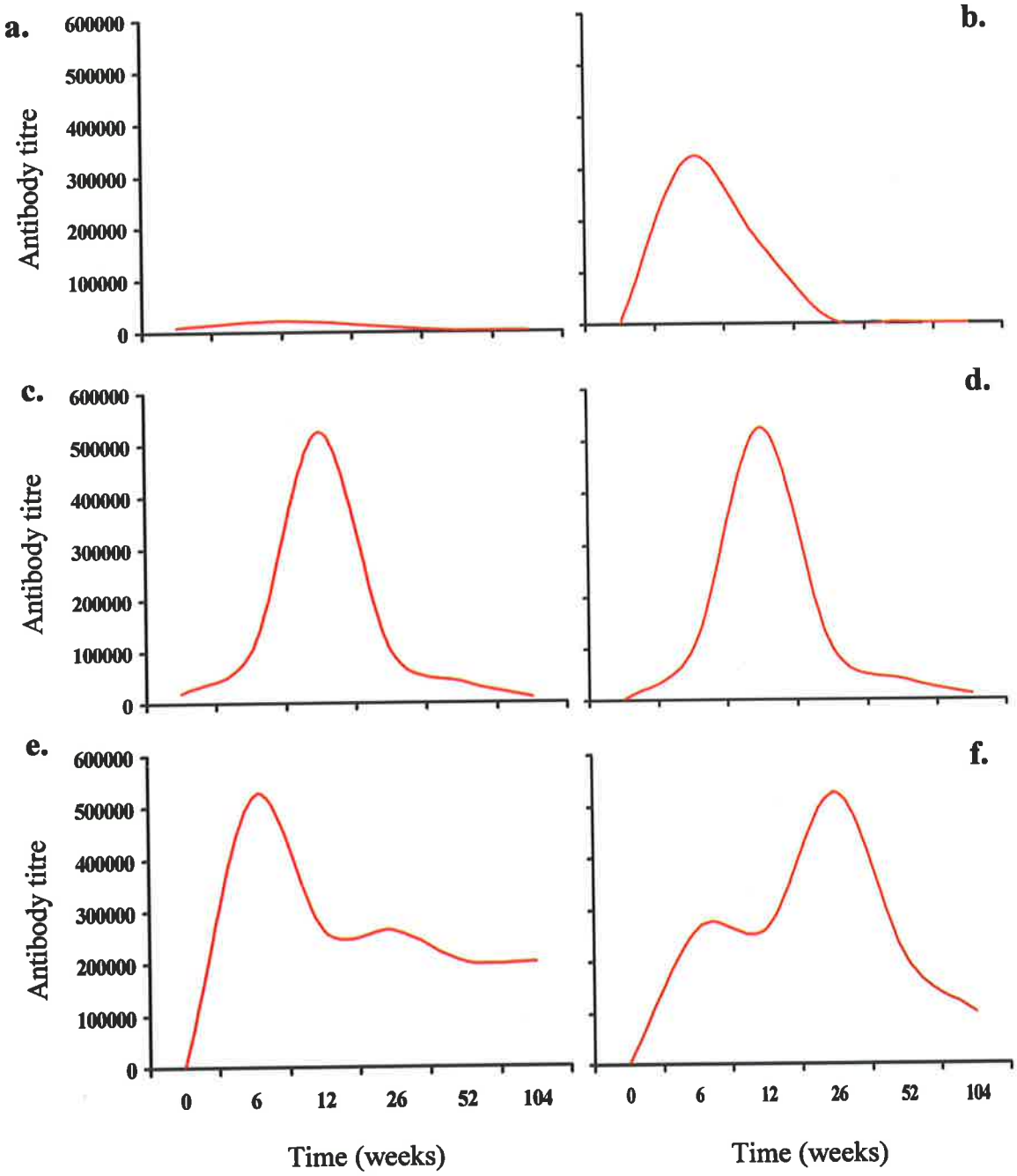

Figure 3.1: Development of antibody titres to rh-α-L-iduronidase in ERT-treated MPS I patients

Sera antibody titres to rh-α-L-iduronidase in enzyme-treated MPS I patients 1-5 and 10 (a) 2 (b), and 6-9 (c-f respectively) over time.

making this mouse model valuable for the study of innate and adaptive immunity. Both the rh-α-L-iduronidase-treated and non-treated Rag-deficient mice only developed low antibody titres to the replacement enzyme (800-3,200), consistent with their inability to mount a proper immune response. In a MPS I mouse model, enzyme administration led to the development of significantly higher antibody titres (12,800 – 819,200) to rh-α-L-iduronidase; considerable variation was observed in the level of antibody titres to rh-α-L-iduronidase between the ERT-treated MPS I mice.

3.1.2 Antibody titres to rh-α-L-iduronidase in ERT treated MPS I patients

Ten MPS I patients receiving ERT (phase I clinical trial) were investigated for immune response to the replacement protein. In all 10 patients prior to the onset of ERT, serum antibody titres to rh-α-L-iduronidase were within the control range. Patient 6 showed the highest level of background antibody titres to α-L-iduronidase (20 000). After the onset of therapy, five out of the 10 patients (patients 2, 6, 7, 8 and 9) developed antibody titres to rh-α-L-iduronidase that were above the normal range (Figures 3.1b-f). Patient 2 had a rapid increase in sera antibodies to rh-α-L-iduronidase by week 6 of therapy followed by a rapid decrease at subsequent time points (Figure 3.1b), characteristic of a transient immune response. The remaining four immune-responsive patients had a more sustained immune response (Figures 3.1c-f) with maximum sera antibodies to rh-α-L-iduronidase seen by weeks 6 to 26 of ERT. Antibody titres gradually decreased at subsequent time points and reached control levels between one- to two-years of therapy. Antibody reactivity was further characterised by epitope mapping.

Table 3.1: Serum antibody titres to α-L-iduronidase in mice

Identification number	Mouse phenotype/ Treatment	Antibody titre
1011	Normal mice/ No enzyme	3 200
1012	Normal mice/ No enzyme	3 200
2221	Immune deficient/ Plus enzyme	1 600
2244	Immune deficient/ Plus enzyme	1 600
2245	Immune deficient/ Plus enzyme	1 600
2246	Immune deficient/ Plus enzyme	800
2249	Immune deficient/ Plus enzyme	3 200
2250	Immune deficient/ Plus enzyme	3 200
2247	Immune deficient/ No enzyme	800
5101	α-L-iduronidase deficient/ Plus enzyme	204 800
5103	α-L-iduronidase deficient/ Plus enzyme	409 600
5104	α-L-iduronidase deficient/ Plus enzyme	819 200
5106	α-L-iduronidase deficient/ Plus enzyme	102 400
5108	α-L-iduronidase deficient/ Plus enzyme	204 800
5109	α-L-iduronidase deficient/ Plus enzyme	51 200
5110	α-L-iduronidase deficient/ Plus enzyme	102 400
5111	α-L-iduronidase deficient/ Plus enzyme	23 600
5112	α-L-iduronidase deficient/ Plus enzyme	204 800
5113	α-L-iduronidase deficient/ Plus enzyme	102 400
5114	α-L-iduronidase deficient/ Plus enzyme	51 200
5115	α-L-iduronidase deficient/ Plus enzyme	102 400
5116	α-L-iduronidase deficient/ Plus enzyme	102 400
5117	α-L-iduronidase deficient/ Plus enzyme	12 800
5118	α-L-iduronidase deficient/ Plus enzyme	204 800
5120	α-L-iduronidase deficient/ Plus enzyme	102 400
5122	α-L-iduronidase deficient/ Plus enzyme	51 200
5125	α-L-iduronidase deficient/ Plus enzyme	51 200
5137	α-L-iduronidase deficient/ No enzyme	400
5138	α-L-iduronidase deficient/ No enzyme	400

3.1.3 Epitope reactivity of ERT treated MPS I patient sera antibodies

Prior to receiving ERT, sera from all but one of the 10 patients displayed little or no epitope reactivity against rh-α-L-iduronidase (Figures 3.2-3.6); the exception, patient 6, showed low-affinity reactivity with several linear peptides spanning the α-L-iduronidase protein sequence (Figure 3.3). All non-immune-responsive MPS I patients (patients 1, 3-5 & 10) displayed little or no epitope reactivity across the α-L-iduronidase protein sequence throughout the duration of ERT (Figure 3.2). Patient 2, who had a transient increase in antibody titres between weeks 6-12, also showed little or no epitope reactivity against α-L-iduronidase (Figure 3.2), indicative of a primary immune response. The remaining four immune-responsive MPS I patients (patients 6-9), showed a similar pattern of epitope reactivity during therapy; by six-weeks of therapy, patients 6-9 displayed low affinity epitope reactivity consistent with the increase in antibody titre seen at this time point (Figures 3.3-3.6). High affinity reactive epitopes were identified by 12-weeks of ERT (Figures 3.3-3.6) followed by a gradual reduction in epitope reactivity at subsequent time points. The pattern and degree of reactivity at 104-weeks of therapy was similar to that seen prior to the start of ERT (Figures 3.3-3.6) and to unaffected human controls.

The linear sequence epitope reactivity for serum antibodies from the four immune-responsive patients (patients 6-9), were mapped onto a linear model of α-L-iduronidase (Figures 3.7b-e). This allowed visualisation of key antibody reactive epitopes on α-L-iduronidase and comparisons between the patients' samples. The crystal structure of α-L-iduronidase has yet to be defined, however, the location of the β-sheets and the probable location of the α-helices have been postulated using bio-informatic techniques. The location of these structures was also depicted on the linear model (Figure 3.7a) to identify

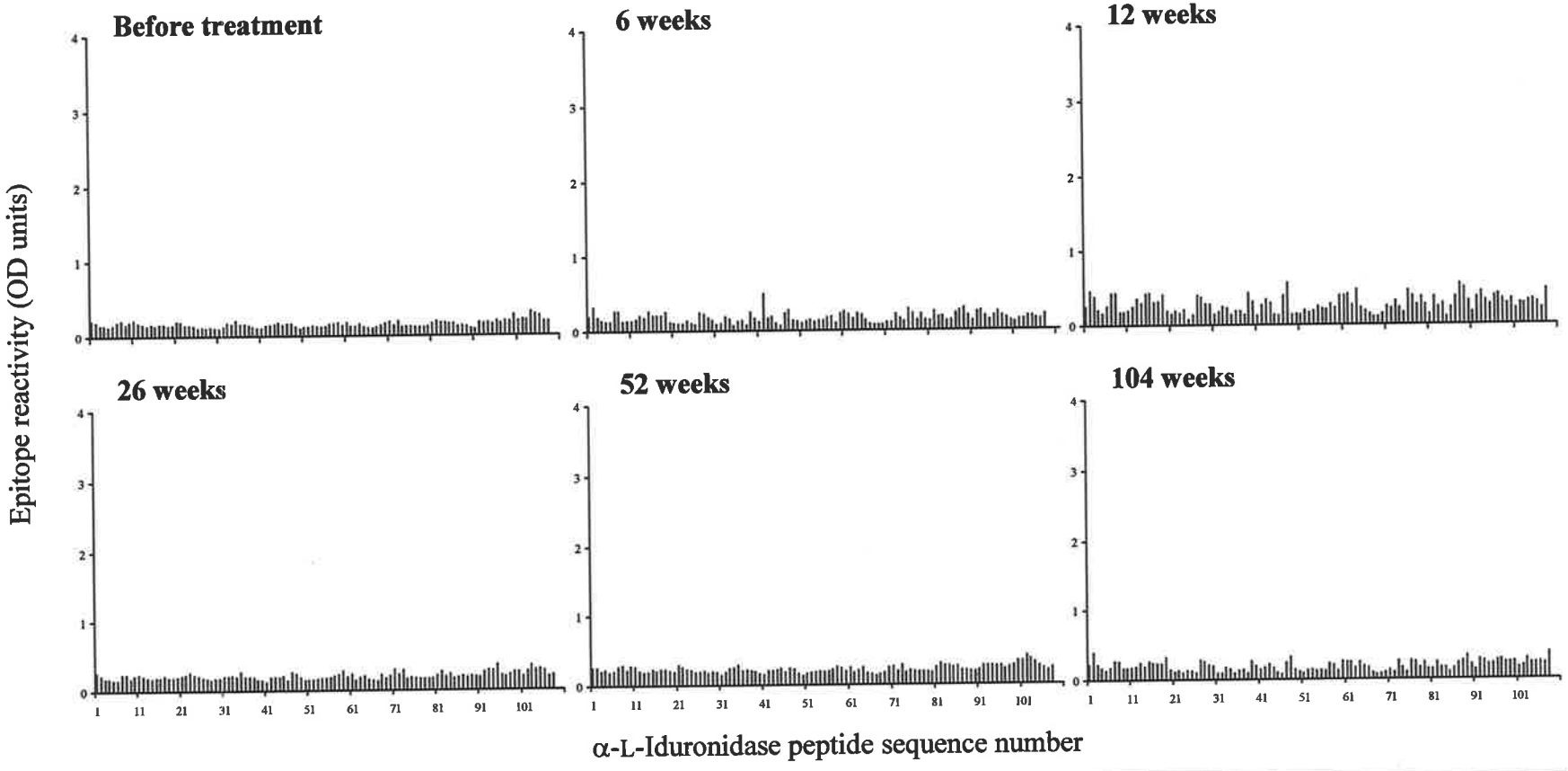

Figure 3.2: Representative pattern of α-L-iduronidase epitope reactivity of serum antibodies from patients 1-5 and 10

Epitope reactivity was expressed as ELISA OD units (y-axis) and was shown for individual α-L-iduronidase peptide sequences (x-axis).

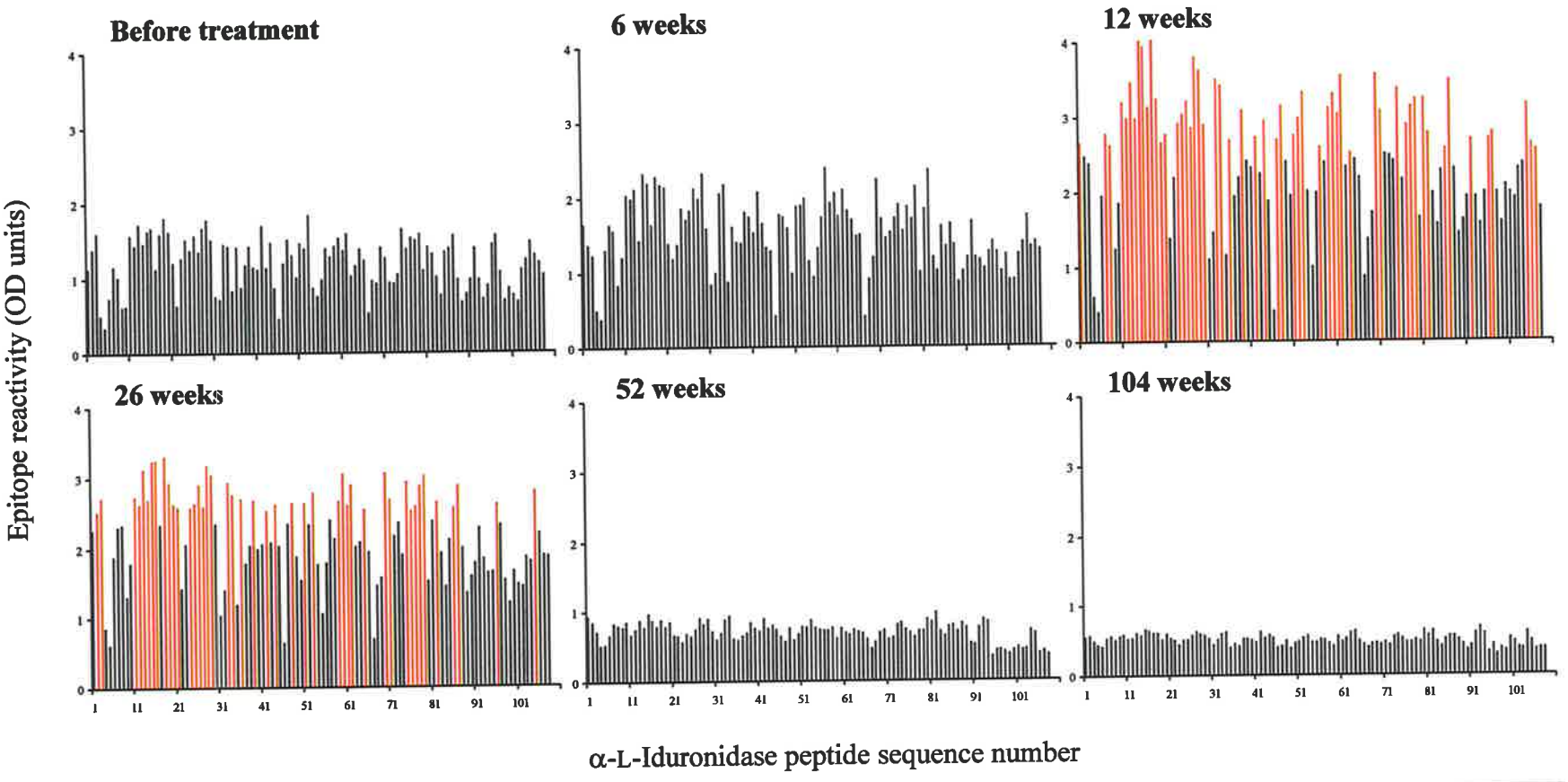

Figure 3.3: α-L-Iduronidase epitope reactivity of serum antibodies from patient 6

Epitope reactivity was expressed as ELISA OD units (y-axis) and was shown for individual α-L-iduronidase peptide sequences (x-axis). Red bars denote high affinity antibody reactivity.

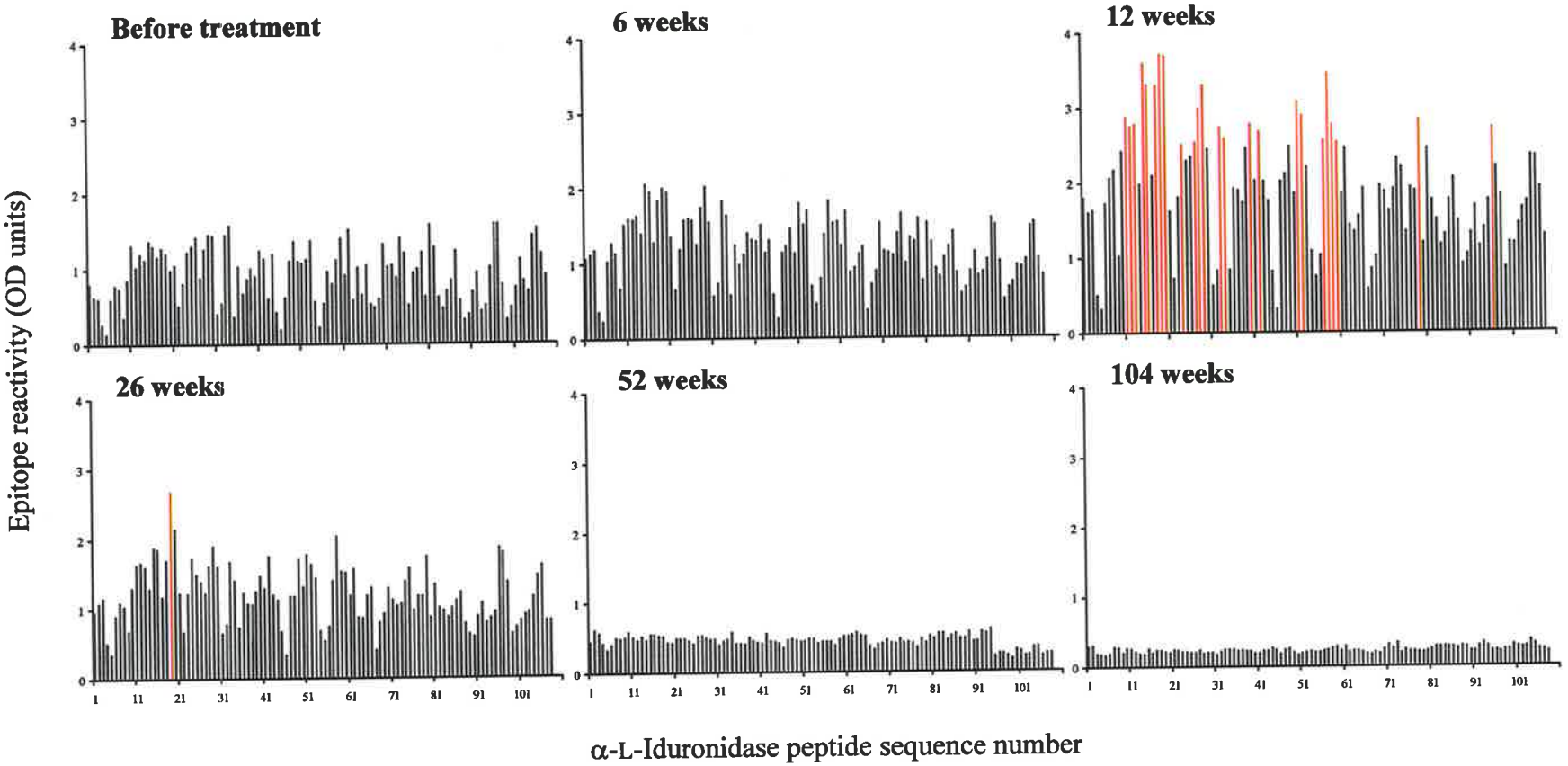

Figure 3.4: α-L-Iduronidase epitope reactivity of serum antibodies from patient 7

Epitope reactivity was expressed as ELISA OD units (y-axis) and was shown for individual α-L-iduronidase peptide sequences (x-axis). Red bars denote high affinity antibody reactivity.

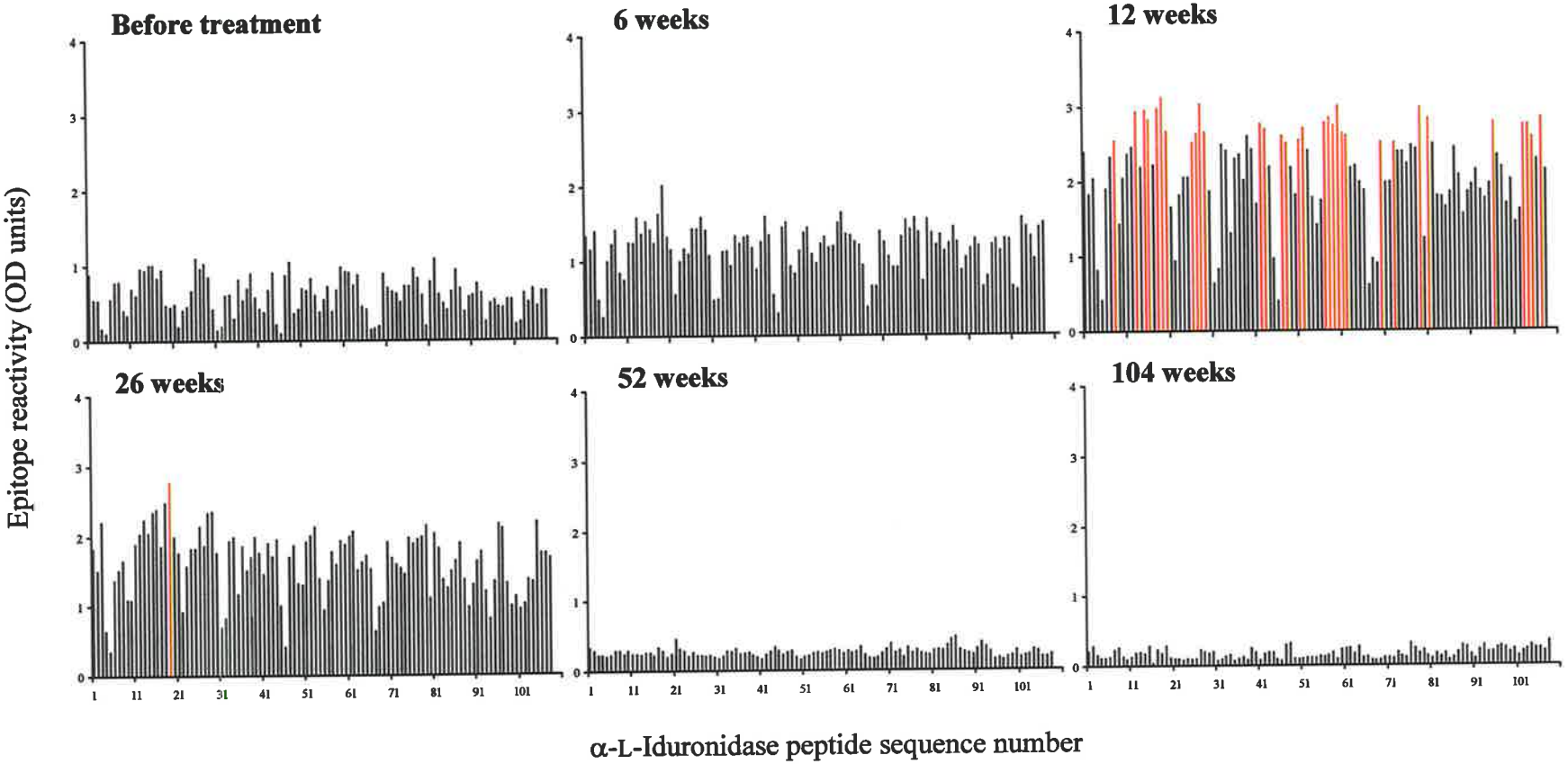

Figure 3.5: α-L-Iduronidase epitope reactivity of serum antibodies from patient 8

Epitope reactivity was expressed as ELISA OD units (y-axis) and was shown for individual α-L-iduronidase peptide sequences (x-axis). Red bars denote high affinity antibody reactivity.

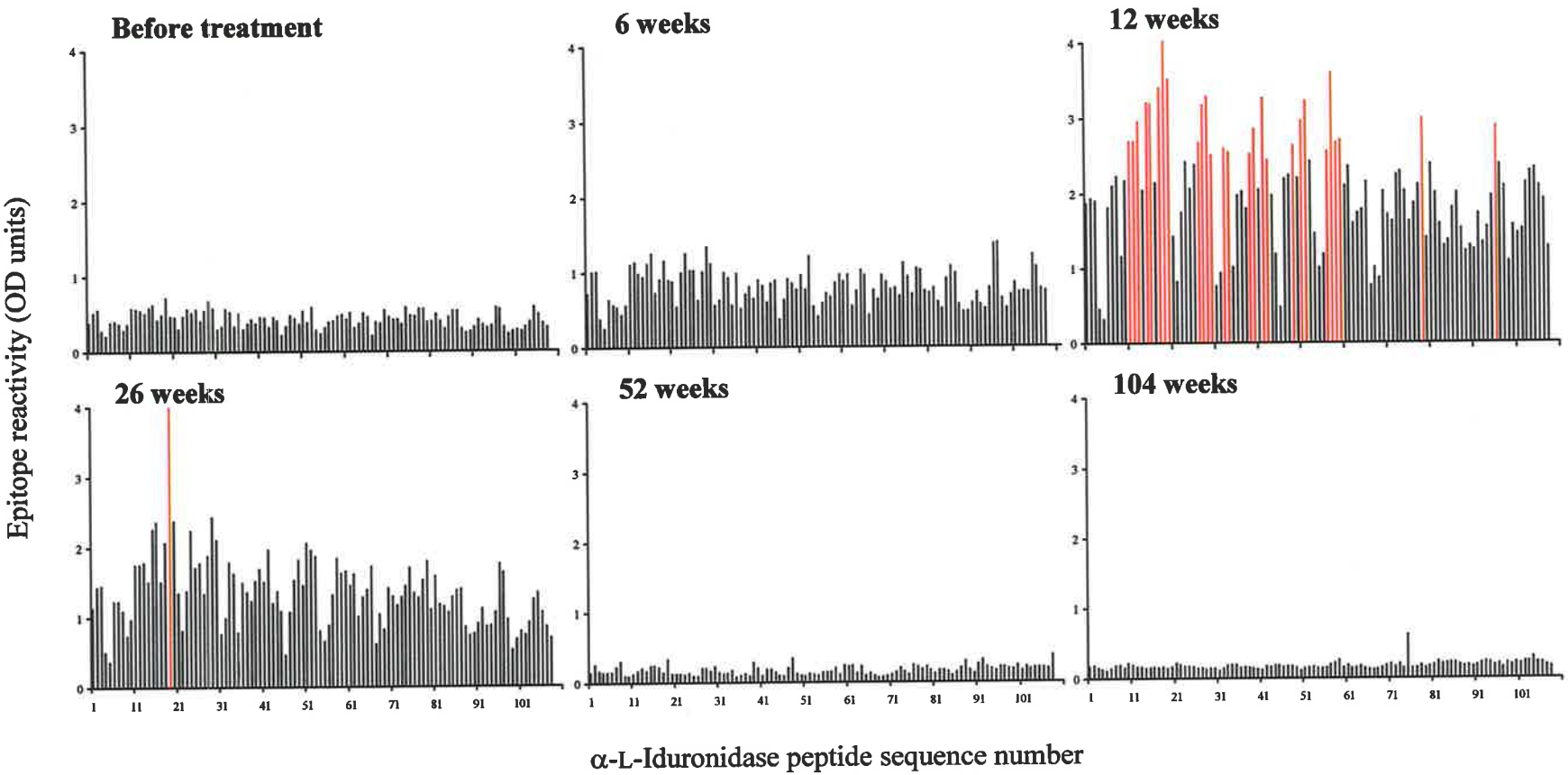

Figure 3.6: α-L-Iduronidase epitope reactivity of serum antibodies from patient 9

Epitope reactivity was expressed as ELISA OD units (y-axis) and was shown for individual α-L-iduronidase peptide sequences (x-axis). Red bars denote high affinity antibody reactivity.

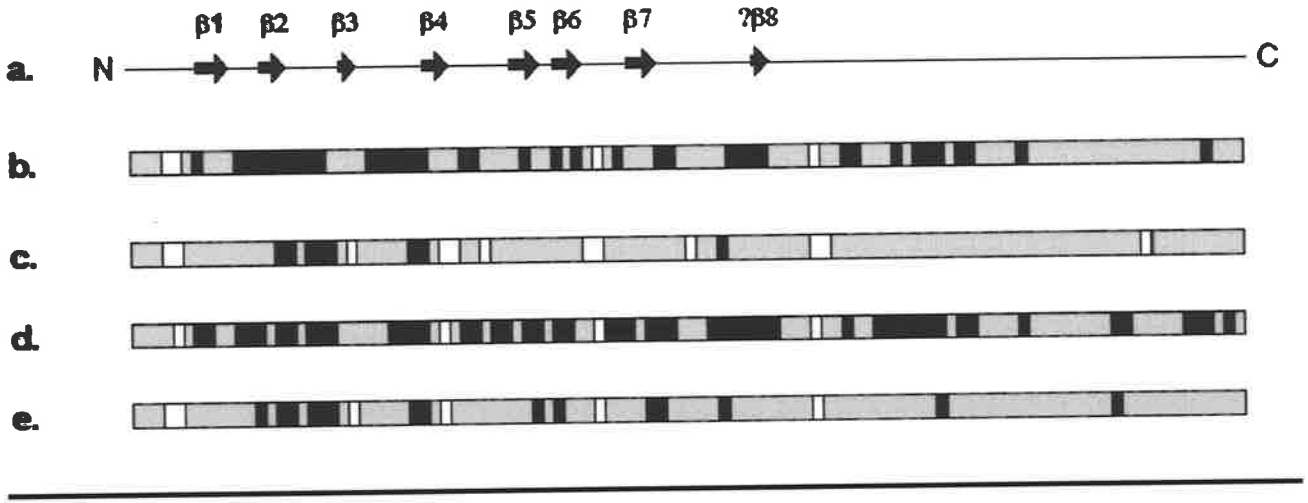

Figure 3.7: Epitope map of antibody reactivity for four immune-responsive ERT-treated MPS I patients

a: α-L-Iduronidase epitopes were mapped onto a linear representation of predicted α-L-iduronidase structural elements. α-L-Iduronidase consists of an (βα) barrel structure active site domain, characteristic of many glycosidases. Arrows represent β-sheet structures (1-7 defined, 8 postulated). The α-helix structures reside between the β-sheet structures; b-e: epitope reactivity of MPS I patients 6-9, respectively; white=no or little antibody reactivity; grey=low-affinity antibody reactivity; black=high-affinity antibody reactivity.

any correlation between antibody-reactive epitopes and the structural elements of the $(\beta/\alpha)_8$ barrel of α-L-iduronidase. For the epitope maps of all four immune-responsive patients, peptides from the first two-thirds of the α-L-iduronidase protein showed higher epitope reactivity with the regions corresponding to the predicted outer α-helix elements and lower reactivity with the predicted internal β-sheet elements, of the $(\beta/\alpha)_8$ barrel. At least 17 peptide sequences on α-L-iduronidase displayed high-affinity reactivity with sera from patients 6-9 at 12-weeks of therapy (Figure 3.7). These sequences were considered important for further investigation. The majority of these peptides mapped to regions between the β-sheets of the $(\beta/\alpha)_8$ barrel, corresponding to the postulated location of the external α-helices. Four of these peptide sequences displayed high-affinity reactivity with sera antibodies from the four immune responsive patients and these were mapped to regions between sheets β_1-β_2, β_2-β_3, β_3-β_4 and at the end of β_8 in the $(\beta/\alpha)_8$ barrel of α-L-iduronidase. One common epitope was peptide 19 (YNFTHLDGYDL) in the α-L-iduronidase sequence, located in the region between the β_2-β_3 sheets, and predicted to be an α-helix element (α2-helix) of the $(\beta/\alpha)_8$ barrel. In three patients (patients 7-9) this site was the last to show high-affinity reactivity in the progression towards immune tolerance (Figures 3.4-3.6). The fourth patient's sera (patient 6) still had reactivity with peptide 19 at 26-weeks of treatment, but also had high affinity reactivity with other peptides (Figure 3.3d). There were also some additional antibody-reactive peptides mapped to the carboxyl end of α-L-iduronidase in all four immune-reactive patients (Figure 3.7).

3.2 Discussion

The development of a humoral immune response to ERT in some MPS I patients and the hypersensitivity reactions reported during clinical trials, led to the investigation of α-L-iduronidase antigenicity and the progression of this antibody response during therapy. This was investigated in both an MPS I animal model and in MPS I patients involved in a phase I clinical trial of ERT.

Immune response to ERT has been reported in several animal models of LSD: in MPS VI cats treated with rh-4-sulphatase, high-affinity antibodies to 4-sulphatase were detected in 30% of animals after 12-months of treatment (Brooks *et al.,* 1997; Turner *et al.,* 1999); in other experiments, 100% of GSD II mice treated with rh-α-D-glucosidase (Raben *et al.,* 2003) and 100% of MPS I dogs treated with either rh- or canine α-L-iduronidase (Kakkis *et al.,* 1996) developed antibody responses to the replacement protein. In this study, we report the antibody response to rh-α-L-iduronidase in a MPS I mouse model. In the absence of a functional immune system (Rag-deficient mice), MPS I mice treated by ERT had antibody titre levels within the control range, similar to the untreated MPS I mice (Table 3.1). However, MPS I mice treated with rh-α-L-iduronidase developed high levels of antibody titres to the replacement protein, ranging from 12,800 to 819,200. Some of this immune response may be attributed to intra-species reactivity. Since MPS I mice have a α-L-iduronidase gene mutation leading to no detectable α-L-iduronidase protein, the variability in antibody response to this replacement protein suggests that other genetic loci or environmental factors may influence the immune response outcome.

Immune responses to ERT have also been reported in human studies (Rosenberg *et al.,* 1999; Amalfitano *et al.,* 2001; Eng *et al.,* 2001; Replagal®, 2001, Aldurazyme®, 2003;

Aryplase®, 2003; Van den Hout *et al.,* 2004; Winkel *et al.,* 2004,). Here, in ERT-treated MPS I patients, those who had an immune response developed high levels of antibody titres by 12-26 weeks of therapy. The sera from these patients showed evidence of the development of numerous high affinity epitopes. The majority of these epitopes mapped to the predicted location of the outer α-helical elements of the $(\beta/\alpha)_8$ barrel structure in α-L-iduronidase (Durand *et al.,* 2000; Brooks *et al.,* 2001). This is not unexpected as the outer α-helices would be more accessible for antibody reactivity than the internal β-sheets. Four different peptide regions showed common reactivity in four of the immune responsive-patients at this time point, with at least 13 other peptide sequences also showing significant reactivity. Peptide 19 on α-L-iduronidase with the sequence YNFTHLDGYDL produced the most sustained epitope reactivity, suggesting a possible role in the development and maintenance of an immune response to ERT in MPS I patients.

MPS I patients who had an immune response showed a decline in both titres and epitope reactivity to α-L-iduronidase after six-months of therapy and developed immune tolerance by one-year of therapy. Peptide 19 on α-L-iduronidase was the last to tolerise, again suggesting a potential role in the immune response to α-L-iduronidase during ERT. α-L-Iduronidase epitopes identified in several different studies were compiled and depicted on a linear model of α-L-iduronidase (Figure 3.8). Peptide 19 of α-L-iduronidase also had reactivity with the monoclonal antibody C5D5, immunised rat sera (Turner *et al.,* 2000) and sera from a patient treated by gene therapy (unpublished results). Peptide 19 is located on the second predicted outer α-helix of the α-L-iduronidase $(\beta/\alpha)_8$ barrel structure (Durand *et al.,* 2000; Brooks *et al.,* 2001). This epitope may represent an important structural element of α-L-iduronidase that is exposed at the surface of the native protein

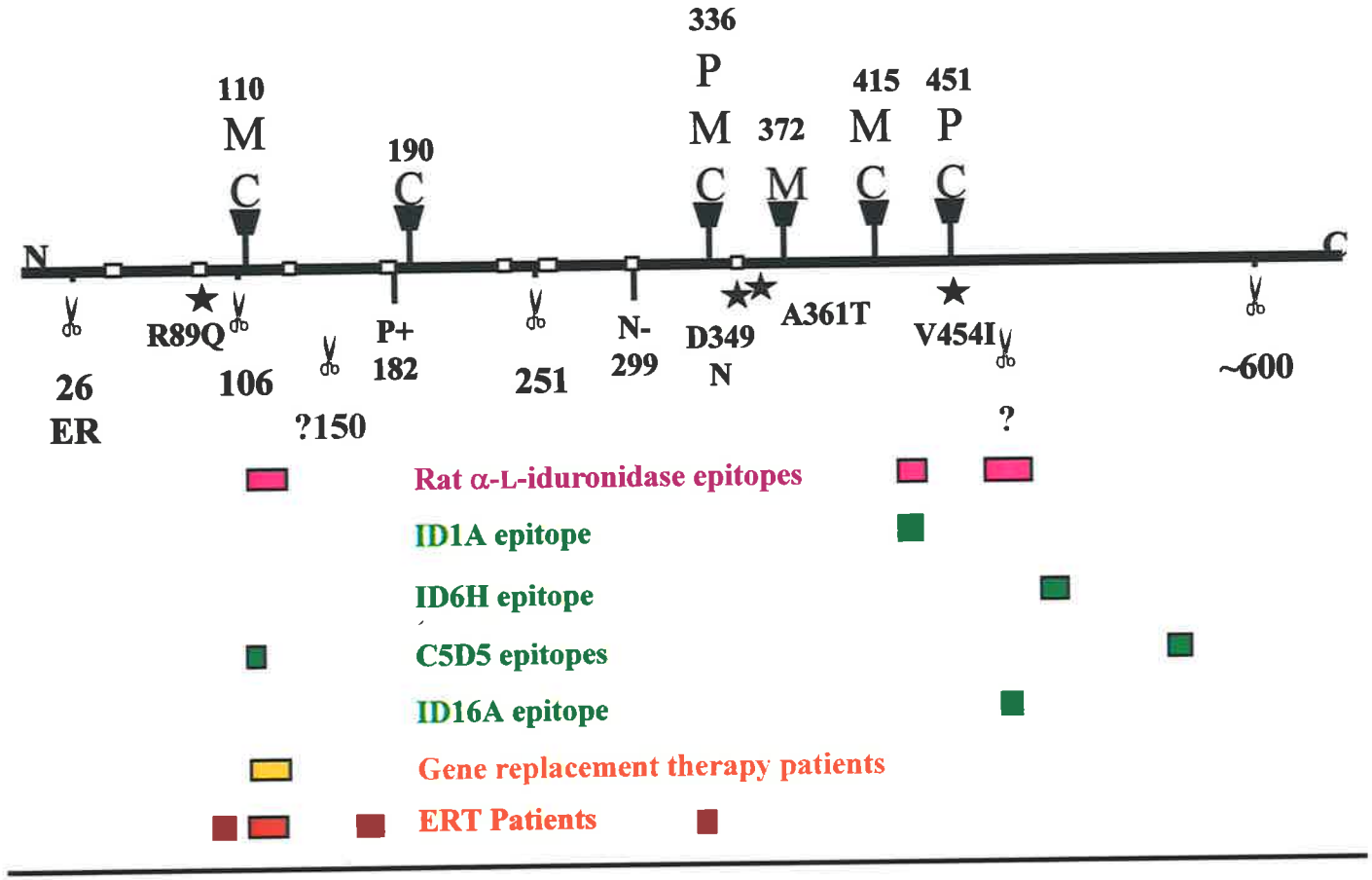

Figure 3.8: α-L-Iduronidase epitope map

A summary of epitopes detected in α-L-iduronidase-immunised rat sera antibodies (■), monoclonal antibodies (■), gene therapy patient sera antibodies (■) and enzyme treated patients (■).

and could also be a structural element of other lysosomal glycosidase enzymes with $(\beta/\alpha)_8$barrel structures (Henrissat *et al.,* 1995).

The reduction in epitope reactivity in sera from MPS I patients, by 104-weeks, had positive implications for patients on long-term ERT as this level of immune reactivity would have minimum impact on the efficacy of therapy. This was supported by a study involving the infusion of rh-α-L-iduronidase into immunised rats, which showed that high antibody titres had an impact on α-L-iduronidase targeting and turnover and that these antibody-mediated effects were absent in rats with titres below 64,000 (Turner *et al.,* 2000). In addition, with the development of immune tolerance, one would expect a reduced risk of antibody-induced hypersensitivity reactions during rh-α-L-iduronidase infusion. A reduced number of clinical signs indicative of hypersensitivity reactions were observed in MPS I patients towards the end of the phase I/II clinical trial of ERT (Kakkis *et al.,* 2001). Recently, a strategy that uses a combination of immune suppressive drugs has been reported to successfully induce immune tolerance in an ERT-treated canine MPS I model (Kakkis *et al.*, 2004). This regimen could be potentially utilised in patients with a problematic immune response to ERT (e.g. neutralising antibodies).

The small sample size of ERT treated MPS I patients did not allow informative conclusions to be made regarding the relationship between genotype and immune response in the enzyme-treated MPS I patients. The fact that all of the immune responsive patients showed multiple epitope reactivity across the α-L-iduronidase protein suggested that patient genotype was not predictive of an immune response. A weak inverse correlation was noted between residual protein concentration detected in skin fibroblast cells (Yogalingam *et al.,* 2004) and the degree of immune response. However, this was not

absolutely predictive as one patient with low protein concentration showed little or no indication of an immune response. The majority of the MPS I patients involved in the phase I/II clinical trial had the more slowly progressing clinical phenotype (Hurler-Scheie and Scheie phenotypes), it would therefore be of particular interest to investigate the humoral immune response to ERT in MPS I patients with null mutations (no protein, Hurler phenotype) that would be expected to mount a more aggressive immune response to ERT. Indeed, all the MPS I mice that have null mutations and therefore have no α-L-iduronidase protein were shown to develop antibody titres to rh-α-L-iduronidase after ERT.

It is evident that some LSD patients receiving ERT will respond with a humoral immune response to the replacement protein. It is important for the treating clinician, to be aware of the progression of this antibody response and to know when immune tolerance develops. This study showed, for the first time, that immune-responsive MPS I patients developed immune-tolerance to rh-α-L-iduronidase after one year of ERT. A specific peptide (peptide 19) in the catalytic domain of the enzyme appeared to be important in the development and maintenance of the immune response to α-L-iduronidase. This epitope was located in the region predicted to be an α-helix element (α2-helix) of the $(\beta/\alpha)_8$ barrel of α-L-iduronidase and may represent an important structural epitope that could also be common to other lysosomal glycosidase enzymes with $(\beta/\alpha)_8$ barrel structures. Therefore, the antigenic comparison of two structurally related lysosomal enzymes was proposed. This consequently formed the basis of Chapter 4.

CHAPTER 4:

GLYCOSIDASE CROSS REACTIVITY

4 Introduction

Glycosyl hydrolases (EC 3.2.1-3.2.3) are a diverse group of enzymes responsible for the hydrolysis of glycosidic bonds. This hydrolysis occurs by an acid catalysis mechanism requiring two critical amino acid residues, a proton donor (also termed acid/base catalyst) and a nucleophile (Sinnott, 1990, McCarter and Withers, 1994). The glycosyl hydrolases have been grouped into more than 90 families based on catalytic activity and sequence similarity (Henrissat, 1991, Henrissat and Bairoch, 1996, Coutinho and Henrissat, 1999) and where available, by structural information. These families have been further categorised into 12 clans, glycosyl hydrolase clan-(GH)-A to GH-L (Davies and Henrissat, 1995; Henrissat *et al.,* 1995; Coutinho and Henrissat, 1999; Rigden *et al.,* 2003).

Despite large differences in size (250-450 residues) and sequence of the catalytic domains (<20% identity), families in clan GH-A show a remarkable conservation in the 3D structure of the active sites (Durand *et al.,* 2000). The common catalytic structural element is a $(\beta/\alpha)_8$ barrel that consists of eight parallel internal β-sheets and eight outer α-helices (Jenkins *et al.,* 1995; Durand *et al.,* 2000; Rigden, 2002). Clan GH-A glycosidases operate with a retaining catalytic mechanism, which requires the proton donor and nucleophile to be respectively located at the C-terminal ends of the fourth and seventh β-sheets of the $(\beta/\alpha)_8$ barrel (4/7 superfamily), with a precise separation of 4.5-5.5Å (McCarter and Withers, 1994; Davies and Henrissat, 1995; Jenkins *et al.,* 1995; Durand *et al.,* 1997). The human lysosomal enzyme α-L-iduronidase is a member of family 39 of glycosyl hydrolases, which belongs to clan GH-A; human α-D-glucosidase is a member of family 31 of glycosyl hydrolases and bioinformatics analysis has indicated that family 31 also belongs to the 4/7 superfamily of $(\beta/\alpha)_8$ barrel glycosidases (Henrissat, 1991; Coutinho and Henrissat, 1999; Durand *et al.,* 2000). The key catalytic amino acid residues have been

identified in human α-D-glucosidase as aspartic acid 518 (nucleophile; Hermans *et al.,* 1991) and aspartic acid 616 (possible proton donor; Okuyama *et al.,* 2001) and in human α-L-iduronidase as glutamic acid 182 (proton donor) and glutamic acid 299 (nucleophile) (Durand *et al.,* 1997; Brooks *et al.,* 2001; Nieman *et al.,* 2003). A deficiency in α-glucosidase causes GSDII while a deficiency of α-L-iduronidase results in MPS I, as discussed in detail in Chapter 1 of this thesis.

ERT has been investigated as a potential therapy for both MPS I (Wraith *et al.,* 2004) and GSD II (Amalfitano *et al.,* 2001; Kishnani and Howell, 2004; Van den Hout *et al,* 2004) patients. A potential complication for ERT is the generation of an immune response to the replacement protein, and this has been reported in both of these patient groups. In GSD II patients 83% have been reported to develop an immune response to ERT [http://www.worldpompe.org/myozyme260405.html] and some patients have developed neutralizing antibodies to rh-α-D-glucosidase (Amalfitano *et al.,* 2001). In MPS I patients, an immune response to rh-α-L-iduronidase has been observed in up to 90% of ERT treated patients (Aldurazyme®, 2003).

It was postulated that the conservation of similar structural features and amino acid motifs in the active sites of glycosidases, using a retaining catalytic mechanism, might result in some common antigenicity. The present study aimed to compare the antigenicity and structural epitopes on the lysosomal glycosidases, α-L-iduronidase and α-D-glucosidase. In Chapter 3, the molecular basis of the humoral immune response to rh-α-L-iduronidase in enzyme-treated MPS I patients was investigated and a specific peptide appeared to be important in the development and maintenance of this antibody reactivity. To determine whether this peptide played a role in the development of the humoral immune response to

ERT in GSD II patients, cross-reactivity studies were performed. The findings have significance for LSD patients being treated by ERT where there is an immune response to a glycosidase replacement protein.

4.1 Results

4.1.1 Epitope reactivity of α-D-glucosidase and α-L-iduronidase polyclonal antibodies

The epitope reactivity of an α-D-glucosidase polyclonal antibody was defined against the full length α-D-glucosidase peptide sequence (Figure 4.1a). High affinity reactivity was demonstrated with a number of peptides across the α-D-glucosidase protein sequence, particularly in peptides 30-120 that were expected to contain the catalytic $(\beta/\alpha)_8$-barrel structural element. The anti-α-D-glucosidase polyclonal antibody showed low level epitope reactivity corresponding to the peptides containing the active site residues (which are located at the end of β-strands forming the $(\beta/\alpha)_8$-barrel) but higher epitope reactivity to adjacent sites (located in the α-helical structures connecting the β-strands in the-barrel). A similar pattern of epitope reactivity was obtained with an anti-α-L-iduronidase polyclonal antibody when mapped onto the α-L-iduronidase protein sequence (Figure 4.1b). There was a trend for the high affinity reactive epitopes located on the first half of the α-L-iduronidase protein sequence (which has been shown to correspond to the $(\beta/\alpha)_8$-barrel) to be present on the outer α-helix elements (Durand *et al.*, 1997). However, this trend was less than previously reported for sera from immune responsive MPS I patients (Kakavanos *et al.*, 2003)

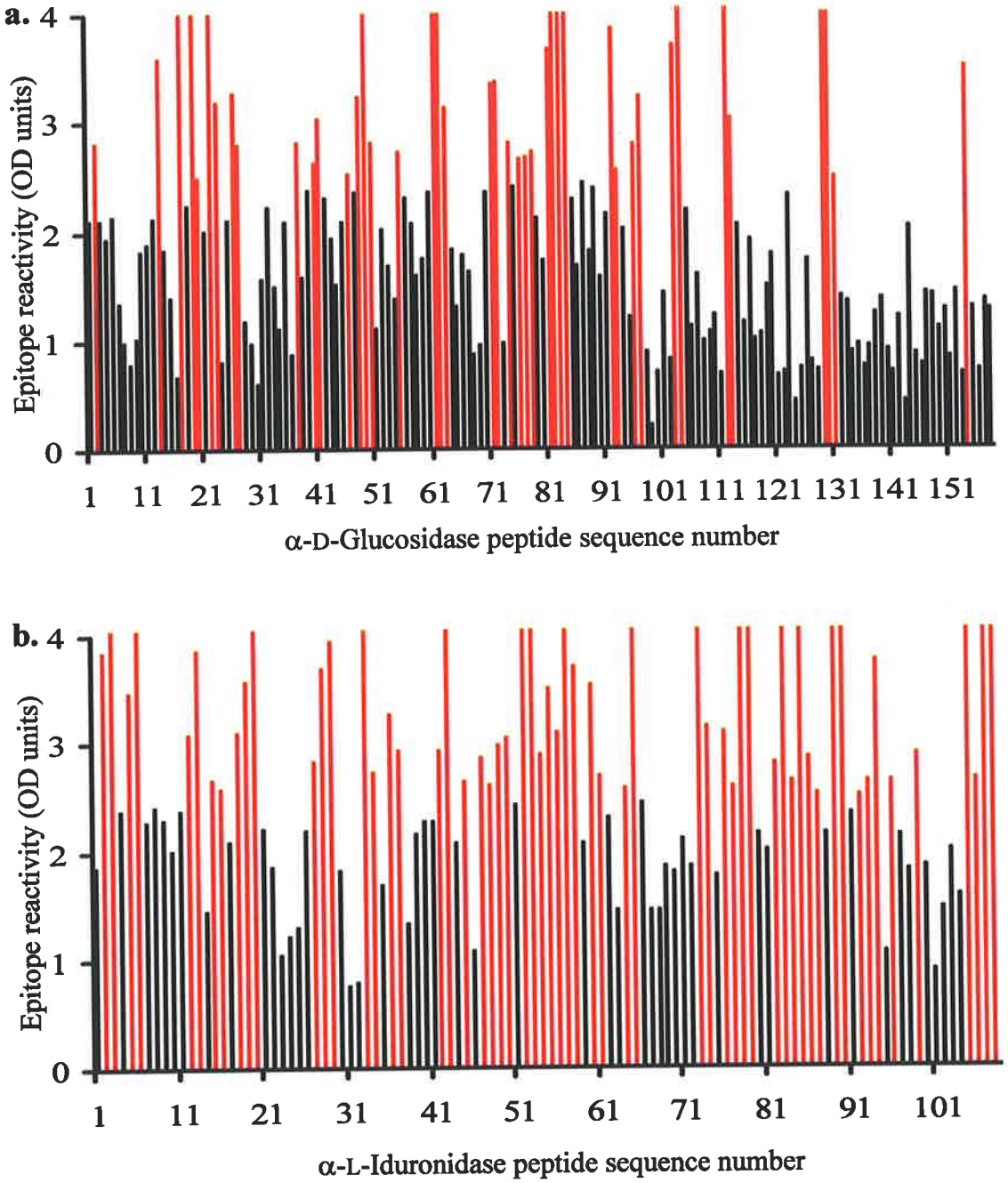

Figure 4.1: α-D-Glucosidase and α-L-iduronidase epitope reactivity of polyclonal antibodies

Epitope reactivity of an anti-α-D-glucosidase polyclonal antibody (a; on α-D-glucosidase peptides) and an anti-α-L-iduronidase polyclonal antibody (b; on α-L-iduronidase peptides). Epitope reactivity was expressed as ELISA OD units (y-axis) and was shown for individual peptide sequences (x-axis). Red bars denote high affinity antibody reactivity.

4.1.1.1 Antibody reactivity to native and denatured forms of rh-α-D-glucosidase

The reactivity of an anti-α-D-glucosidase polyclonal antibody and the monoclonal antibodies 73A1, 81D3, 81A6, 43D1, 118G3 and 43G8, was evaluated against native, semi-denatured (boiled) and denatured (boiled with β-mercaptoethanol) rh-α-D-glucosidase (Figure 4.2). The anti-α-D-glucosidase polyclonal antibody displayed high reactivity with native and semi-denatured forms of rh-α-D-glucosidase and slightly less reactivity with the denatured rh-α-D-glucosidase. This corresponded well with the number of high-affinity linear sequence epitopes on α-D-glucosidase seen with the polyclonal antibody (Figure 4.1a). The monoclonal antibodies 81D3 and 81A6 also reacted equally with the native and semi-denatured forms of rh-α-D-glucosidase, but had slightly less reactivity with denatured rh-α-D-glucosidase (Figures 4.2c-d). The monoclonal antibodies 73A1 and 43D1 had similar reactivity to both native and denatured rh-α-D-glucosidase (Figures 4.2b,e), which indicated that the epitopes recognised by these two antibodies were to exposed linear sequence epitopes. It was interesting to note that 118G3 had lower reactivity with semi-denatured rh-α-D-glucosidase compared with the denatured rh-α-D-glucosidase, which inferred that the epitope was to exposed linear sequence but also had a conformational component. Unlike the other monoclonal antibodies, 43G8 displayed low reactivity even with the native form of rh-α-D-glucosidase (Figure 4.2f). Denaturation of rh-α-D-glucosidase resulted in complete loss of reactivity with the monoclonal antibody 43G8, indicating the conformational nature of the epitope.

4.1.2 Epitope reactivity of monoclonal antibodies to α-D-glucosidase

The monoclonal antibody 43D1 demonstrated high affinity reactivity with peptides 86 and 87 on the α-D-glucosidase protein sequence (Figure 4.3a). These peptides contained the

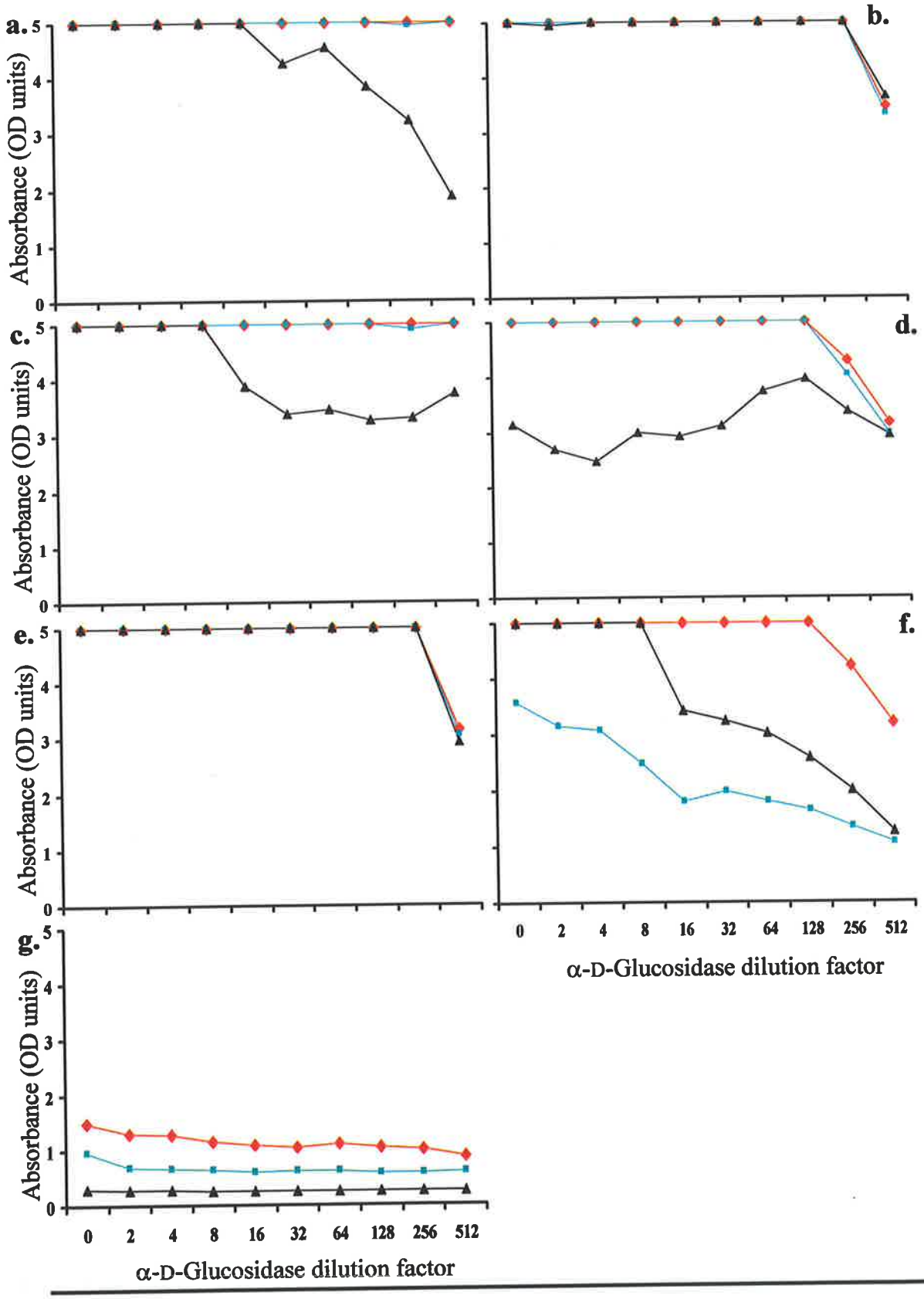

Figure 4.2: Antibody reactivity against α-D-glucosidase

Reactivity of an anti-α-D-glucosidase polyclonal antibody (a) and monoclonal antibodies 713A1, 81D3, 81A6, 43D1, 118G3 and 43G8 (b-g respectively), against native (◆), semi-denatured (■) and denatured (▲) α-D-glucosidase.

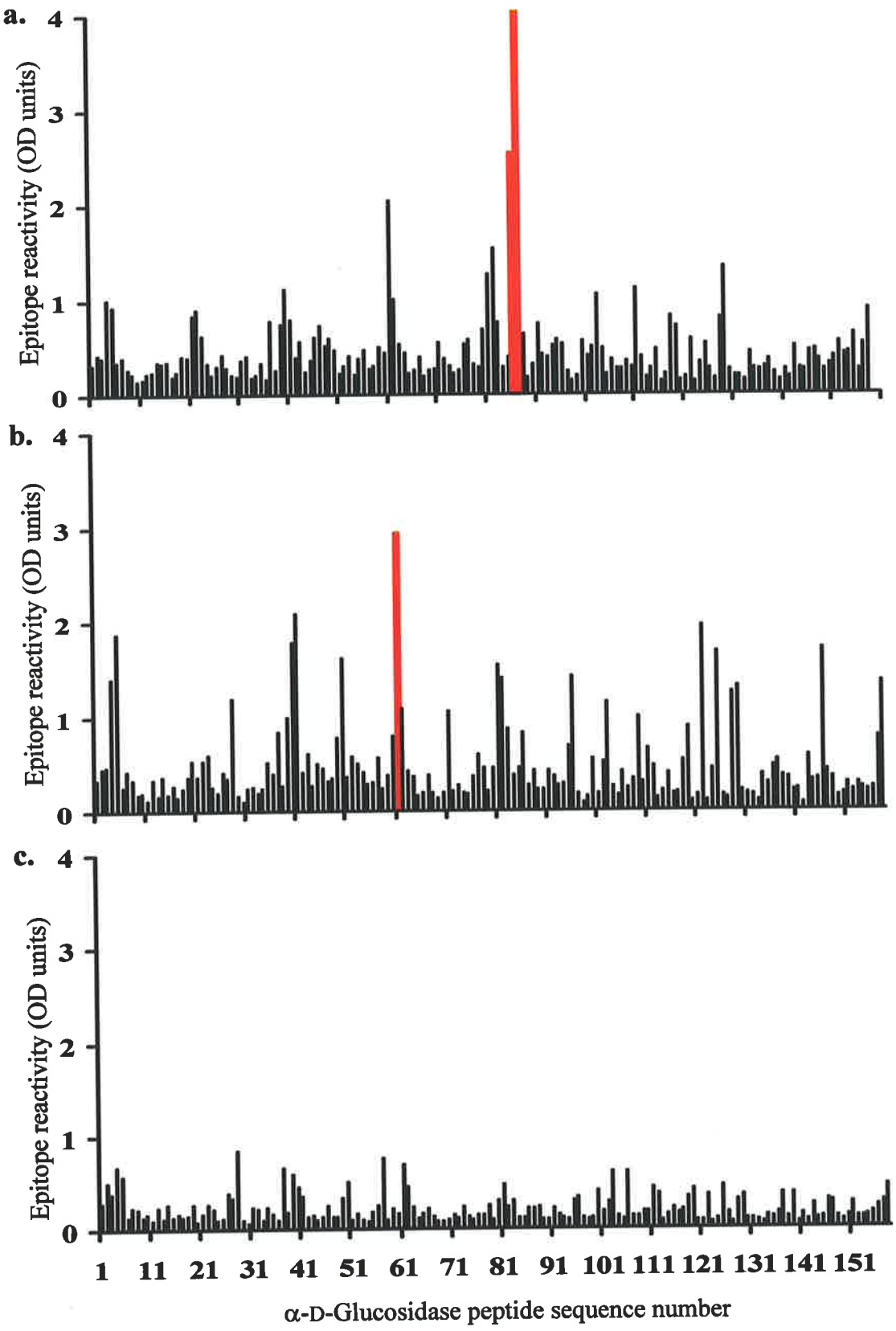

Figure 4.3: Epitope reactivity of monoclonal antibodies against α-D-glucosidase

The monoclonal antibodies 43D1 (a), 118G3(b) and 43G8 (c) were depicted with the level of ELISA OD reactivity shown on the y-axis and peptide sequence numbers on the x-axis. Red bars denote high affinity antibody reactivity.

nucleophilic aspartic acid residue of α-D-glucosidase (active site), which would be predicted to localise to the β-4 sheet of the $(\beta/\alpha)_8$-barrel. The monoclonal antibody, 43D1, also demonstrated low affinity reactivity with peptide number 61. Another monoclonal antibody, 118G3, demonstrated reactivity with peptide number 61 of the α-D-glucosidase protein sequence and low affinity reactivity with a number of other peptides across the α-D-glucosidase protein (Figure 4.3b). The monoclonal antibody, 43G8, showed no specific epitope reactivity and was presumed to react with a conformational epitope (Figure 4.3c). The monoclonal antibody, 81D3, demonstrated high affinity reactivity with peptide 20 on the α-D-glucosidase protein sequence (Figure 4.4a). This antibody was representative of two other monoclonal antibodies, 73A1 and 81A6, which showed similar epitope reactivity with this peptide (Figures 4.4b-c). The latter epitope was located at one of the known proteolytic processing sites (Moreland *et al.,* 2005) on α-D-glucosidase.

4.1.3 Effect of temperature on monoclonal antibody reactivity to rh-α-D-glucosidase

Temperature treatment of precursor rh-α-D-glucosidase had little or no effect on epitope reactivity for the monoclonal antibodies 81D3 and 43D1 (Figure 4.5a). The monoclonal antibody, 118G3, showed a temperature-dependant reduction in reactivity with progressive heat denaturation of precursor rh-α-D-glucosidase (>50°C; Figure 4.5a). The monoclonal antibody, 43G8, showed minimal reactivity with precursor rh-α-D-glucosidase, but some reactivity after heat treatment at 50°C, followed by progressive reduction in reactivity at higher temperatures (Figure 4.5a).

The monoclonal antibody 81D3, had only minimal reactivity with mature processed rh-α-D-glucosidase, consistent with its location at a proteolytic processing site (Figure 4.5b).

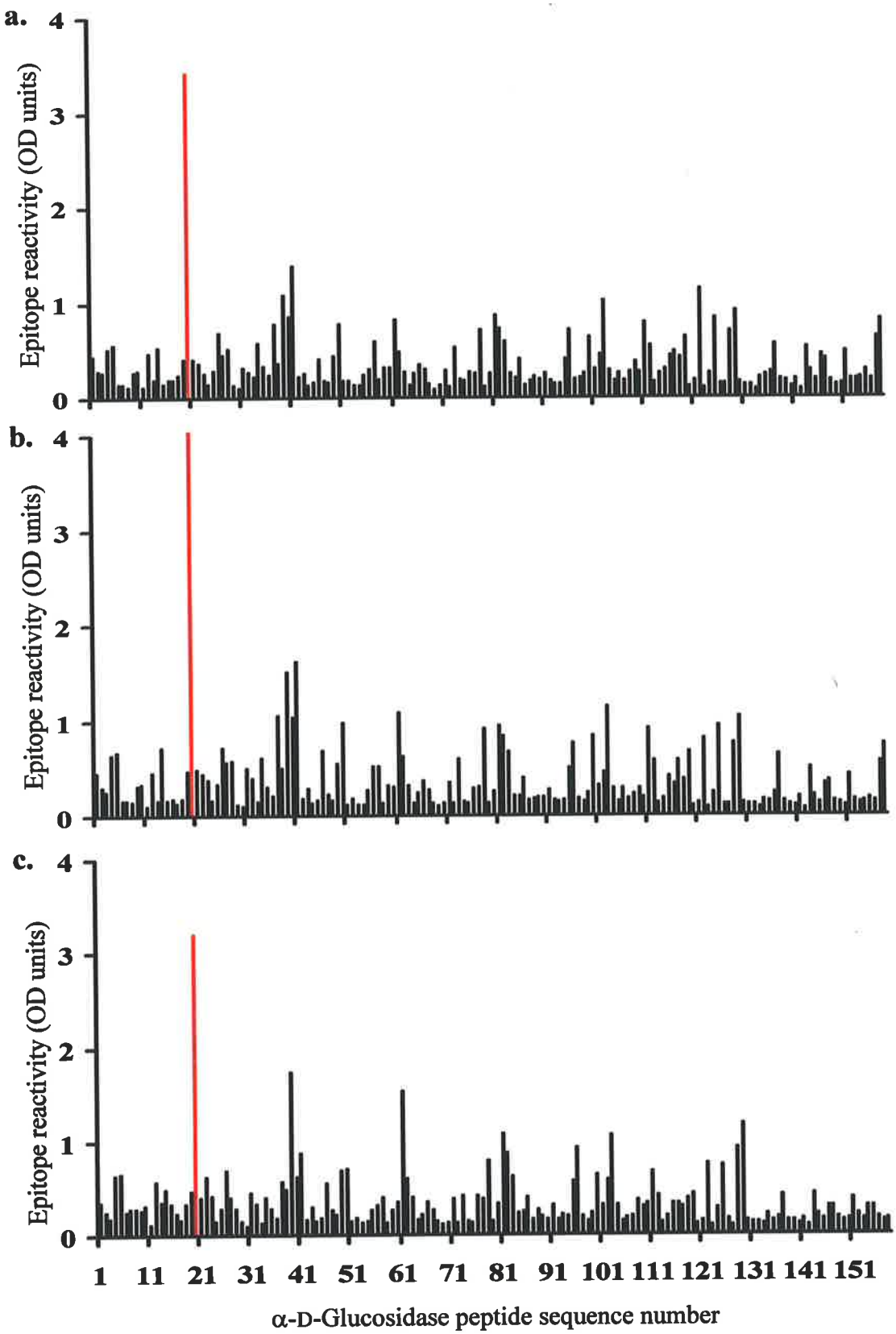

Figure 4.4: Epitope reactivity of monoclonal antibodies against α-D-glucosidase

The monoclonal antibodies 81D3 (a), 73A1 (b) and 81A6 (c) were depicted with the level of ELISA OD reactivity shown on the y-axis and peptide sequence numbers on the x-axis. Red bars denote high affinity antibody reactivity.

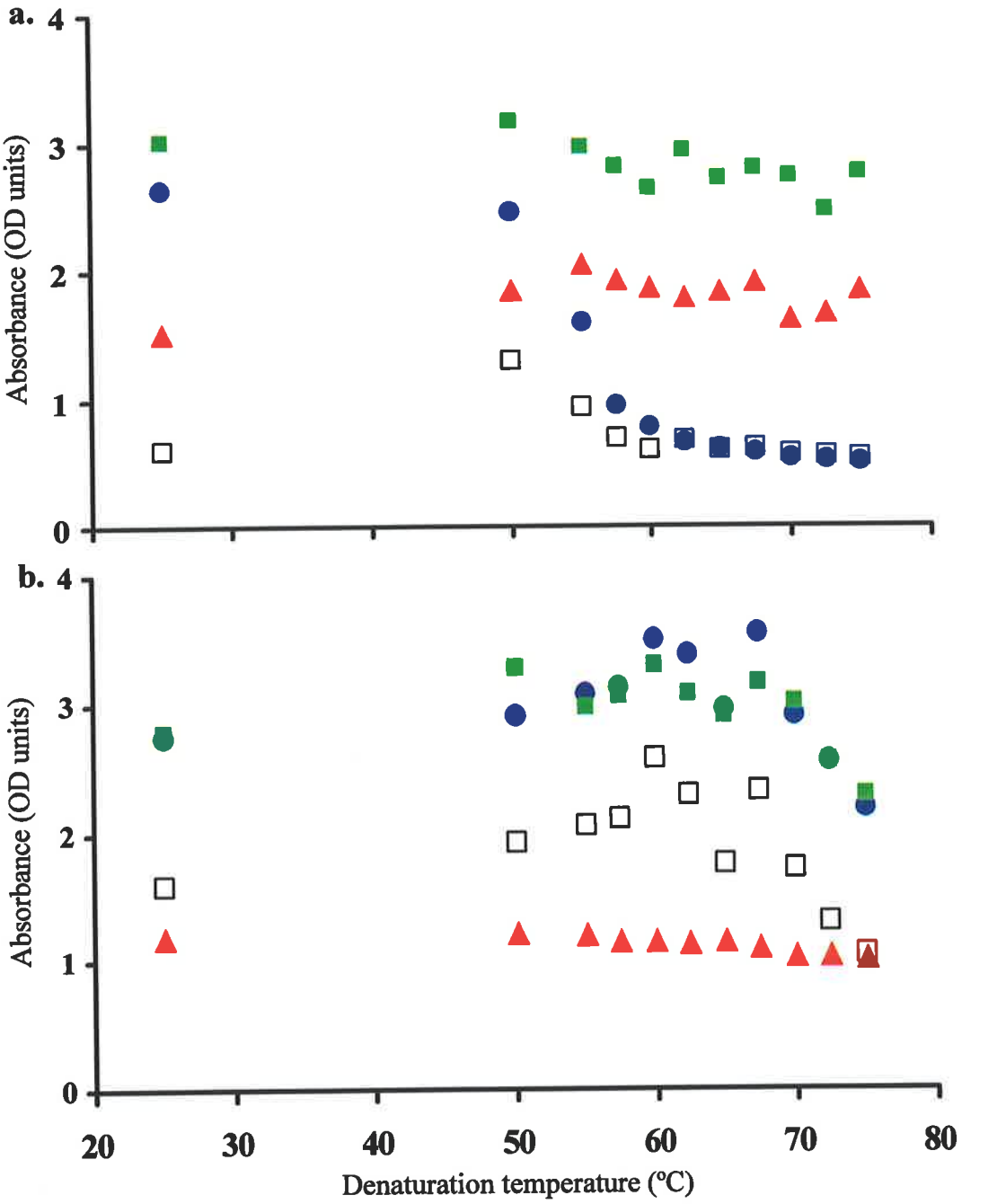

Figure 4.5: The effect of heat treatment on the reactivity of monoclonal antibodies against either secreted precursor (a) or intracellular proteolytically processed α-D-glucosidase (b). The monoclonal antibodies were depicted as 43D1 (■), 81D3 (▲), 118G3 (●), and 43G8 (□). The y-axis shows the level of ELISA OD reactivity plotted against the temperature of heat treatment (x-axis).

In contrast, 118G3 showed reactivity with processed rh-α-D-glucosidase and consistent reactivity with heat treatment up to 65°C (Figure 4.5b). Above this temperature a temperature-dependent decrease in 118G3 reactivity was observed, which was similar to that produced by the monoclonal antibodies 43D1 and 43G8 (Figure 4.5b). These studies suggested that precursor and proteolytically processed α-D-glucosidase had different antigenic properties and different responses to denaturation.

4.1.4 Antibody titres to rh-α-D-glucosidase in four immunised rats

Four Sprague Dawley male rats were evaluated for antibody production after immunisation with rh-α-D-glucosidase, purified from transgenic rabbit milk (Pharming BV, The Netherlands). The non-immunised controls displayed low levels of serum antibody titres to rh-α-D-glucosidase of 200-800 (Figure 4.6). In contrast, the immunised rats had significantly higher serum antibody titres to rh-α-D-glucosidase, ranging from 204,800 to 409,600 (Figure 4.7). Antibody titres to denatured rh-α-D-glucosidase were almost identical to those for the native protein (Figure 4.8), which inferred that the majority of immune reactivity among the immunised rats was to linear sequence epitopes. This allowed further characterisation of the antibody reactivity by epitope mapping.

4.1.4.1 Epitope reactivity of immunised rat sera antibodies

Sera from the non-immunised controls displayed little or no epitope reactivity against α-D-glucosidase but also showed low-affinity reactivity with a few linear peptides spanning the α-D-glucosidase protein sequence (Figure 4.9). Immunised rat sera displayed both low epitope reactivity with the majority of the linear peptides spanning the α-D-glucosidase protein, and also high-affinity reactivity with multiple peptides (Figure 4.10, Table 4.1). Sera antibodies from immunised rat 1 demonstrated high affinity reactivity with peptides

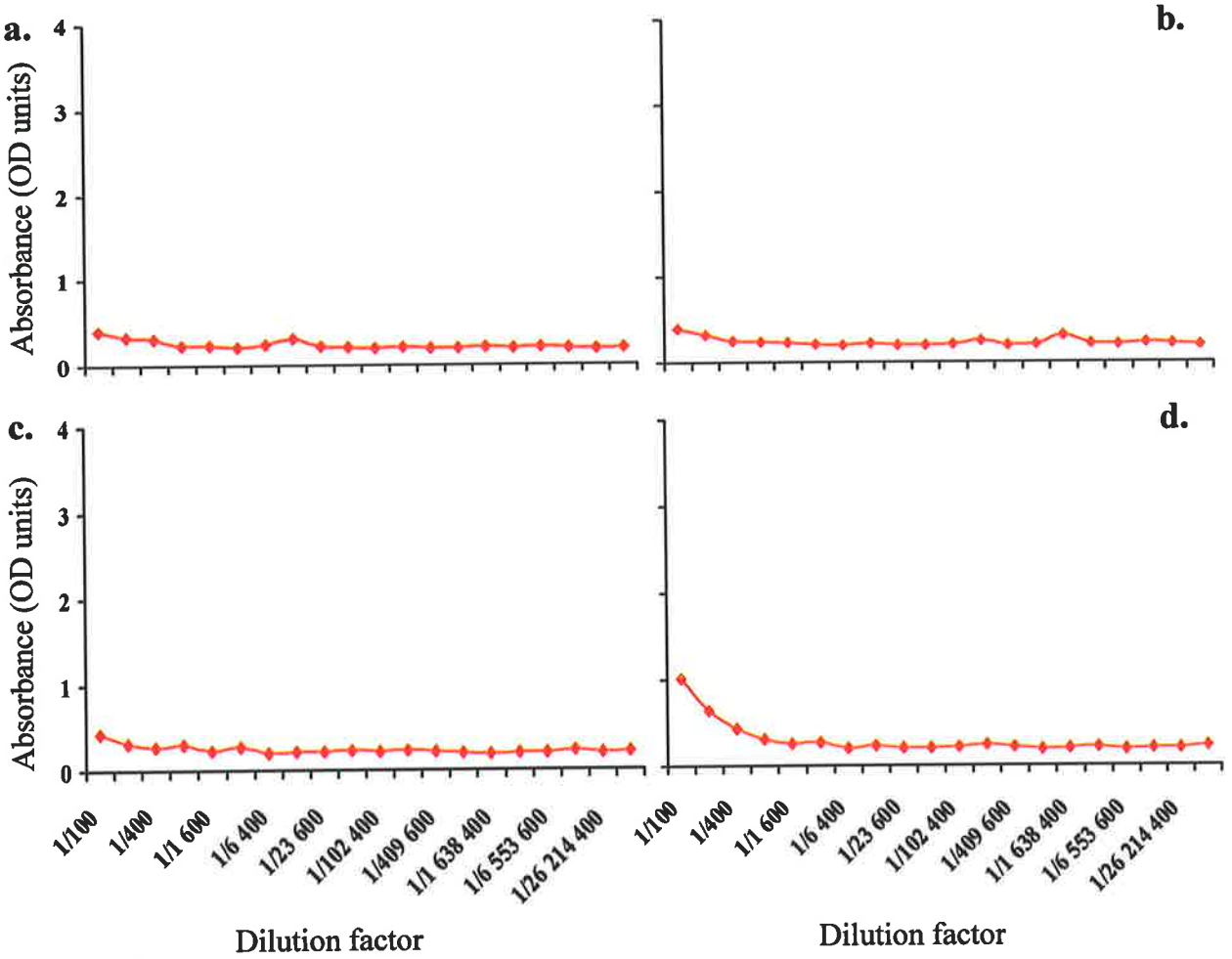

Figure 4.6: Serum antibody titres to α-D-glucosidase in four control rats

Control rats did not receive α-D-glucosidase. Antibody titres from four different rats were depicted (a-d respectively) and defined as the lowest dilution of plasma sample at which the optical density at 410nm was greater than two standard deviations above background i.e. 200 (a), 200 (b), 200 (c) and 800 (d).

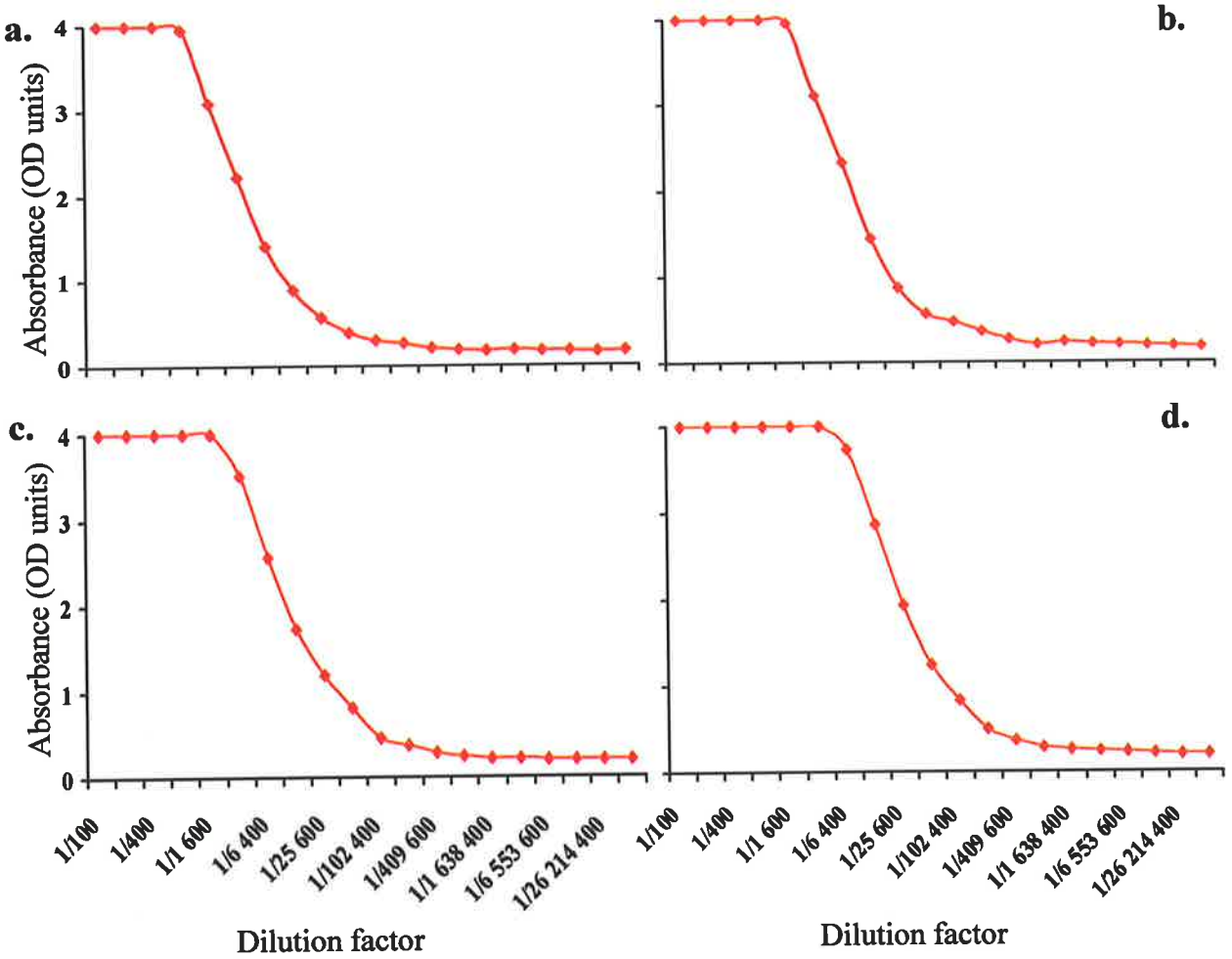

Figure 4.7: Serum antibody titres to α-D-glucosidase in four immunised rats

Immunised rats received 100 μg of α-D-glucosidase fornightly for eight-weeks. Antibody titres post-immunisation from four different rats were depicted (a-d respectively) and defined as the lowest dilution of plasma sample at which the optical density at 410 nm was greater than two standard deviations above background i.e. 204 800 (a & b), 409 600 (c & d).

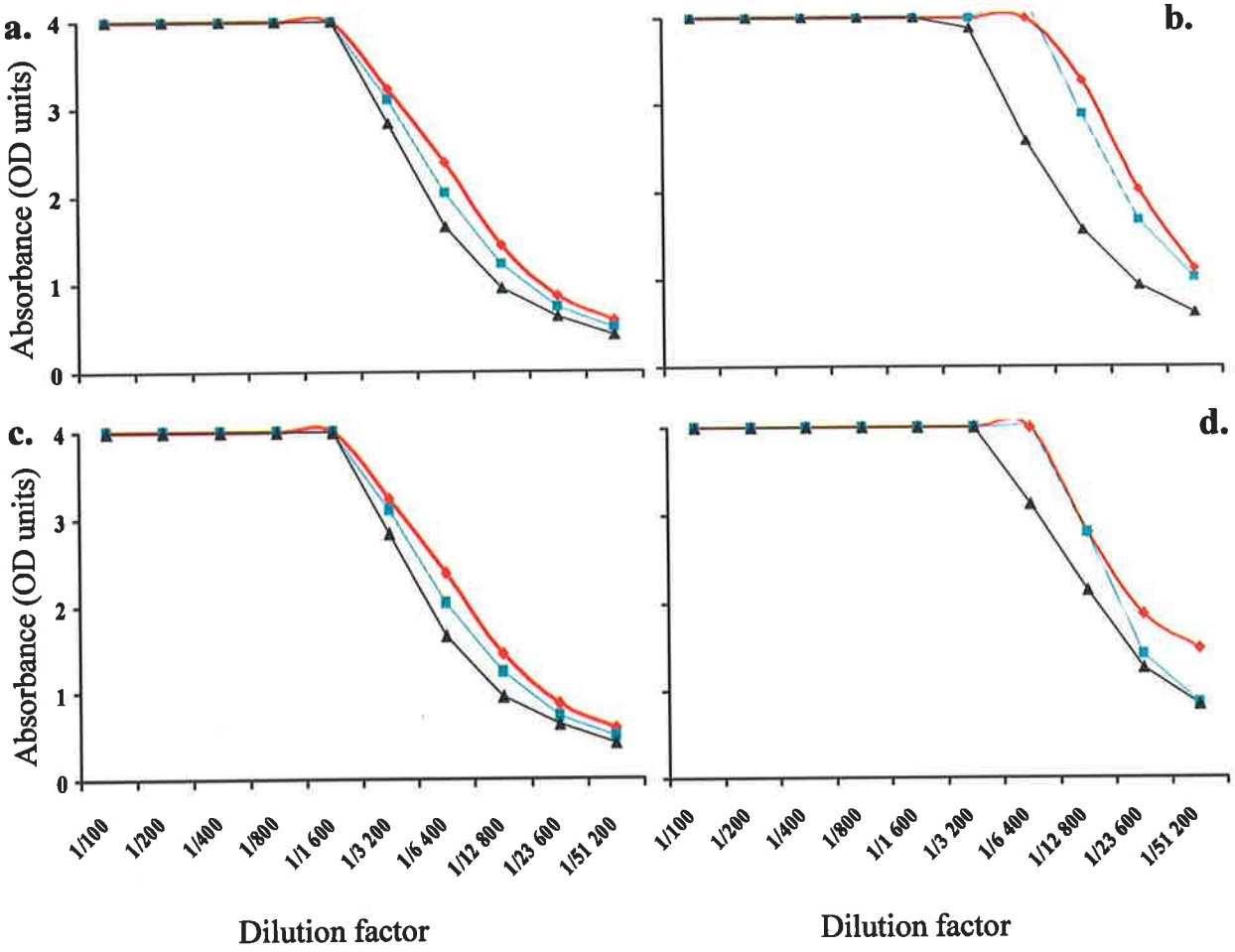

Figure 4.8: Immune reactivity of rat sera antibodies to denatured α-D-glucosidase

Four immunised rats (a-d respectively) received 100 μg of α-D-glucosidase fornightly for eight-weeks. Antibody reactivity post-immunisation against native (◆), semi-denatured (■) and denatured (▲) α-D-glucosidase are depicted.

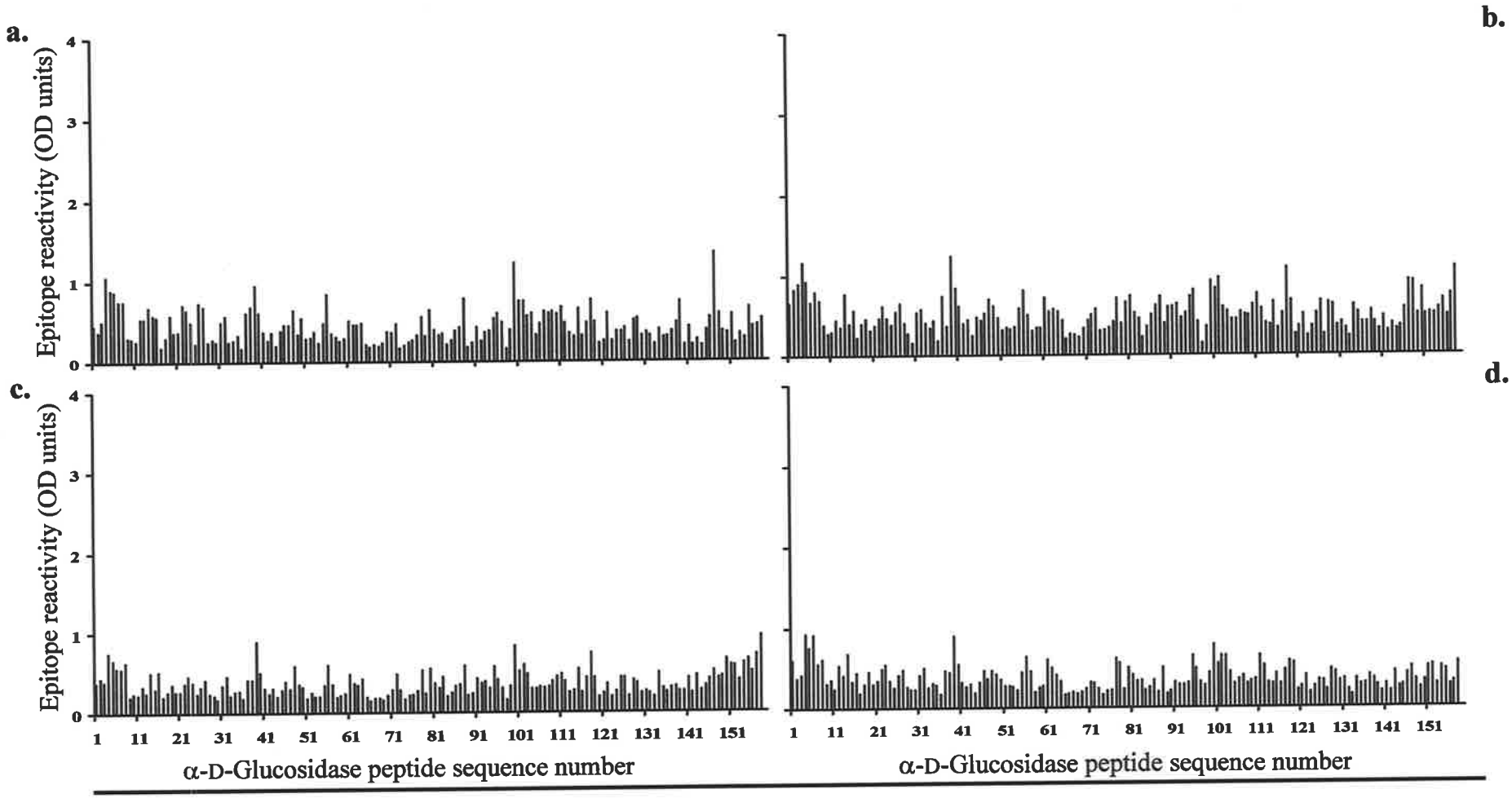

Figure 4.9: α-D-Glucosidase epitope reactivity of serum antibodies in four control rats

Epitope reactivity in control rats 1-4 (a-d respectively) to α-D-glucosidase was expressed as ELISA OD units (y-axis) and was shown for individual peptide sequences (x-axis). Red bars denote high affinity antibody reactivity.

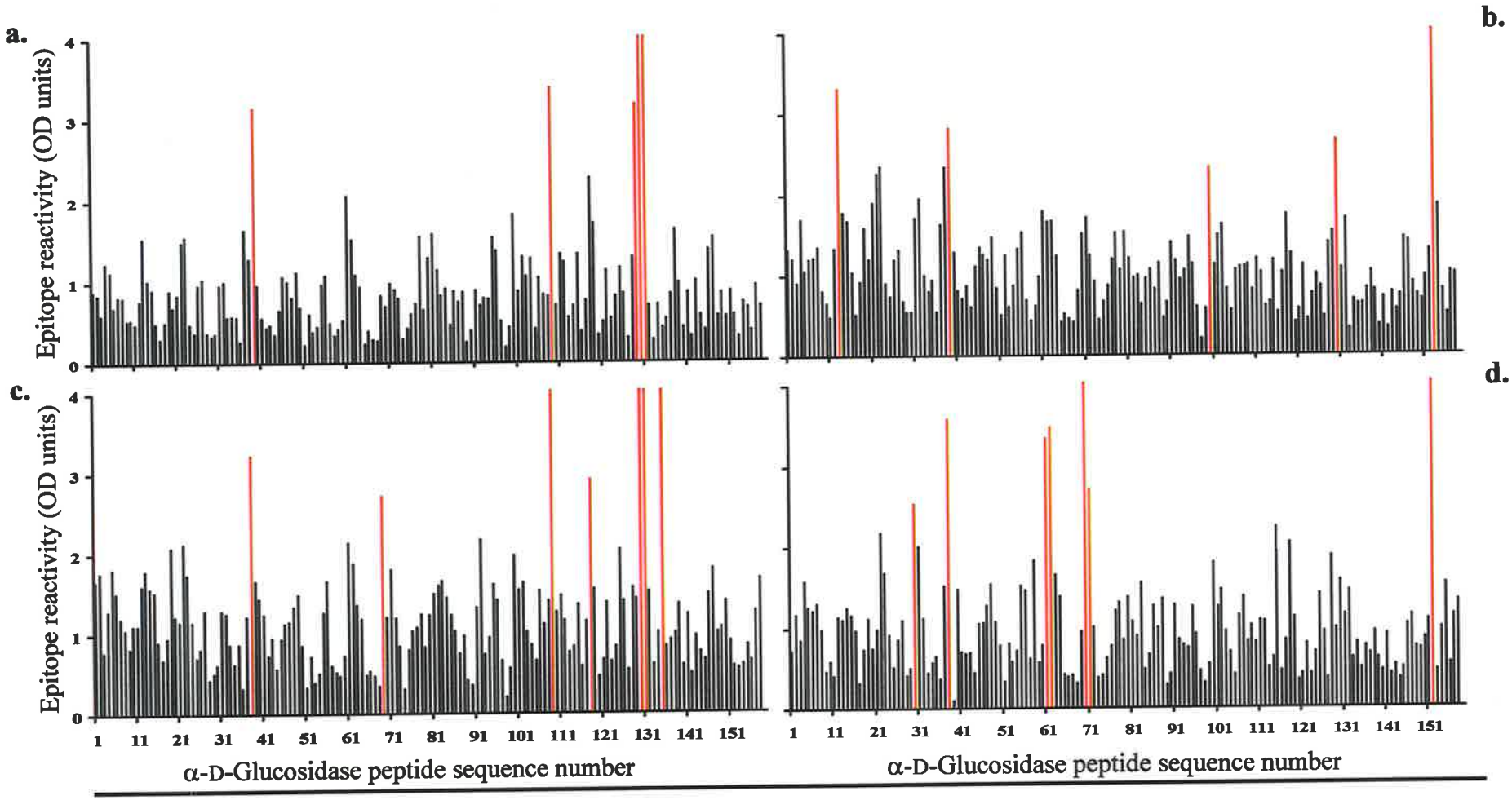

Figure 4.10: α-D-Glucosidase epitope reactivity of serum antibodies in four immunised rats

Epitope reactivity in immunised rats 1-4 (a-d respectively) to α-glucosidase was expressed as ELISA OD units (y-axis) and was shown for individual peptide sequences (x-axis). Red bars denote high affinity antibody reactivity.

Table 4.1 : High affinity linear sequence epitopes in α-D-glucosidase immunised rats

α-D-Glucosidase Peptide No	Peptide Sequence	Sequence Location	Function of Residues
13	GRPRAVPTQCDV	73-84	P-trefoil domain/ Disulphide bond C82
30	TENRLHFTIKDP	175-186	76kDa GAA
37	PFGVIVHRQLDG	217-228	70kDa GAA
38	HRQLDGRVLLNT	223-234	70kDa GAA/ Carbohydrate 233
39	RVLLNTTVAPLF	229-240	70kDa GAA
61	LDVVGYPFMPPY	355-366	β1 sheet?
62	PFMPPYWGLGFH	361-372	β1 sheet?
69	DSRRDFTFNKDG	409-420	end of β2
70	TFNKDGFRDFPA	415-426	between β2- β3
71	FRDFPAMVQELH	421-432	between β2- β3
100	PFVISRSTFAGH	595-606	end of β5
109	FLGNTSEELCVR	649-660	end of β7 / Carbohydrate 652
118	YALLPHLYTLFH	703-714	after β8
129	LGTWYDLQTVPI	769-780	-
130	LQTVPIEALGSL	775-786	-
131	EALGSLPPPPAA	781-792	-
135	VTLPAPLDTINV	805-816	-
152	LGVATAPQQVLS	907-918	-

37, 109, 129, 130 and 131 on the α-D-glucosidase protein sequence (Figure 4.10a); sera antibodies from immunised rat 2 demonstrated high affinity reactivity with peptides 13, 39, 100, 130 and 153 (Figure 4.10b); immunised rat 3 had high affinity reactivity with peptides 38, 69, 109, 118, 130, 131, and 135 (Figure 4.10c); and immunised rat 4 had similar reactivity with peptides 30, 38, 61, 62, 70, 71 and 152 (Figure 4.10d). For some of these high-affinity epitopes the positional location of antibody reactivity was partially coincident for the different rat sera, but the precise linear sequence epitopes varied between rats. The region consisting of peptides 37-39 on the α-D-glucosidase protein sequence was common to all four immunised rats; the region of peptides 129-131 was common to three of the four immunised rats; and the regions 69-71, 109 and 152-153 were common to two of the four rats. The sequences and functions of the α-D-glucosidase residues corresponding to these regions were as depicted in Table 4.1. The antigenic sites were mainly located at the ends of the β-sheets of the $(\beta/\alpha)_8$ barrel of α-D-glucosidase, which would correspond to α-helical elements, a trend also seen with the other glycosidase, α-L-iduronidase (section 3.1.3, Kakavanos *et al.,* 2003). A number of antigenic sites were also identified upstream and downstream of the $(\beta/\alpha)_8$ barrel of α-D-glucosidase.

4.1.5 Epitope cross-reactivity of α-D-glucosidase and α-L-iduronidase antibodies

An anti-α-D-glucosidase polyclonal antibody demonstrated strong cross-reactivity with peptides 19 (YNFTHLDGYLDL) and 83 (PVFPTAEQFRRM) from the α-L-iduronidase protein sequence (Figure. 4.11b); these two peptides also showed high affinity reactivity with the anti-α-L-iduronidase polyclonal antibody (Figure 4.1b), confirming the antigenicity of these sites. Similarly, the anti-α-L-iduronidase polyclonal antibody showed high affinity reactivity with peptide 37 (PFGVIVFRQLDG), on the α-D-glucosidase

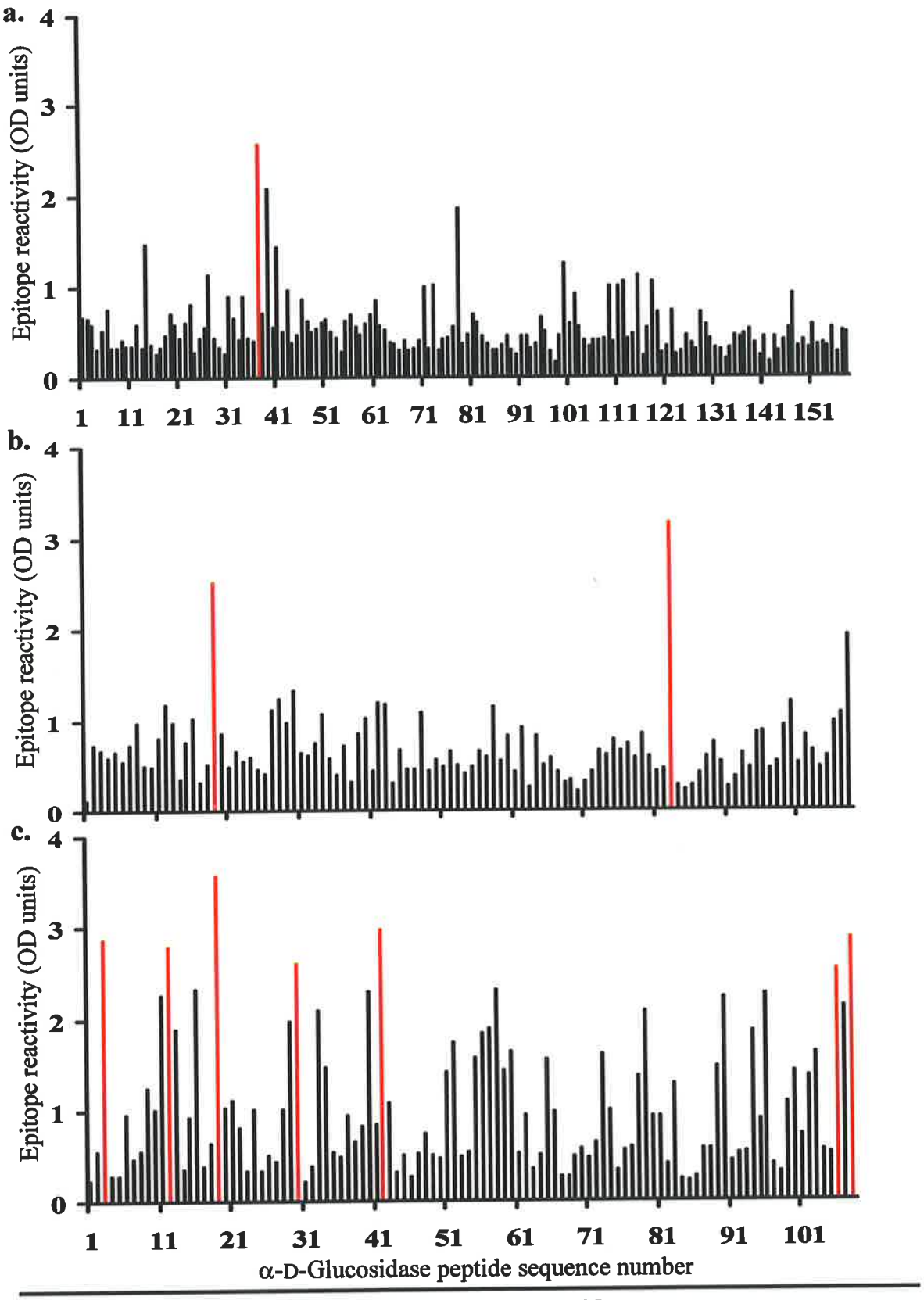

Figure 4.11: Epitope cross-reactivity between glycosidases

Epitope reactivity of an anti-α-L-iduronidase polyclonal antibody when tested against α-D-glucosidase peptides (a). Epitope reactivity of an anti-α-D-glucosidase polyclonal antibody (b) and the α-D-glucosidase monoclonal antibody 43D1 (c) when tested against α-L-iduronidase peptide sequences. The y-axis shows the level of ELISA O.D. reactivity plotted against the peptide sequences (x-axis). Red bars denote high affinity antibody reactivity.

protein sequence and a lower affinity reactivity with several other linear sequence peptides (Figure 4.11a). Some sequence identity was noted between peptide 19 on α-L-iduronidase and peptide 37 on α-D-glucosidase, with a common LDG sequence motif (Table 4.2).

The α-D-glucosidase monoclonal antibody 43D1 demonstrated some cross-reactivity with several epitopes across the α-L-iduronidase protein sequence (Figure 4.11c), including peptides 3 (LLASLLAAPVA), 12 (SWDQQLNLAYVG), 19 (YNFTHLDGYLDL), 30 (WNEFTWNEPDHH), 42 (FFTGEAGVRLDL), 106 (PFSDPVPYLEVP) and 108 (PVPRGPPSPGNP). None of the five other α-D-glucosidase monoclonal antibodies showed detectable cross-reactivity with the α-L-iduronidase protein sequence (data not shown). Similarly, three monoclonal antibodies to α-L-iduronidase failed to react with α-D-glucosidase protein sequence (data not shown).

Sera antibodies from an ERT-treated MPS I patient (JAN 007) demonstrated cross-reactivity with peptide 55 (SPALSWRSTGGI) from α-D-glucosidase (Figure 4.12a) and this was an epitope detected by the anti-α-D-glucosidase polyclonal antibody (Figure 4.1a). Sera from two other MPS I patients who generated an immune response to ERT did not produce cross-reactivity with the α-D-glucosidase peptide sequence (Figures 4.12 b-c).

4.1.6 Analysis of antigenic motifs that had sequence identity

From the cross-reactive α-D-glucosidase and α-L-iduronidase epitopes, four amino acid sequence motifs were identified: NEP, LDG, PTA and SPAL (Table 4.2). A search of the Swiss-Protein database revealed that these sequences were present in 18 other glycosidases (Table 4.2). Although all four sequences were found in α-D-glucosidase and α-L-iduronidase, only one or two of the sequences were found in the other glycosidases.

Table 4.2: Sequence identity motifs in glycosidase enzymes

Enzyme	EC No. (family)	Catalytic Activity	Sequence	Sequence Location	Function of residues	Location/ Disease
Lactase-phlorizin hydrolase	3.2.1.108 3.2.1.62 **Family 1**	Lactose	KFWMTF**NEP**MYLAWL ALKAYR**LDG**IDLRGY KFWITL**NEP**FVIAYQ	1064-1066 1305-1307 1537-1539	proton donor 1065 proton donor 1538	Brush border/ Disaccharide intoler. III
β-glucuronidase	3.2.1.31 **Family 2**	DS and KS	SRECKE**LDG**LWSFRA VMWSVA**NEP**ASHLES	41-43 450-452	β-sheet proton donor 451	Lysosomal/ MPS VII
β-mannosidase	3.2.1.25 **Family 2**	β-D-mannose	MTVLFE**LDG**GLNIEK	309-311		Lysosomal/ CNS myelin
α-N-acetylgalactos-aminidase	3.2.1.49 **Family 27**	N-acetyl-D-galactosamine	KVDMLK**LDG**CFSTPE	155-157	Nucleophile 156	Lysosomal/ Schindler
Glucosylceramidase	3.2.1.45 **Family 30**	D-glucosyl-N-acylsphingosine	WAVTAE**NEP**SAGLLS	273-275	Proton donor 274?	Lysosomal/ Gaucher
α-D-glucosidase	3.2.1.20 **Family 31**	1,4- glucose	VIVHRQ**LDG**RVLLNT DVVLQP**SPAL**SWRST FPDFTN**PTA**LAWWED GMWIDM**NEP**SNFIRG	226-228 325-328 493-495 520-523	 nucleophile 518	Lysosomal/ GSD II
Maltase-glucoamylase	3.2.1.30 **Family 31**	1,4-D-glucose	PFTPRI**LDG**YLFCKT GMWIDM**NEP**SSFVNG	558-60 1421-1423	Active site 1422, nucleophile 1419	Brush border membrane
Sucrase-isomaltase	3.2.1.48 3.2.1.10 **Family 31**	sucrose and maltose	GLWIDM**NEP**SSFVNG	1395-1397	nucleophile 1393, active site 1396	Brush border/ Disaccharide intoler. I
Sialidase 1	3.2.1.18 **Family 33**	sialic acids	QGSTWS**PTA**FIVNDG	123-125		Lysosomal/ Sialidosis
Sialidase 2	3.2.1.18 **Family 33**	Sialic acids	VVAQAR**LDG**HRSMNP	79-81		Cytosolic
α-mannosidase IIx & 2C1	3.2.1.1114 3.2.1.24 **Family 38**	α-D-mannose $Man_5(GlcNAc)_3$	LQLQLG**LDG**HRTLPS TFFWEG**LDG**SRVLVH	724-726 415-417		Golgi ?
Epididymis-specific α-mannosidase	3.2.1.24 **Family 38**	α-D-mannose	RHYSIR**PTA**GAQEGT	544-546		Secreted
α-mannosidase	3.2.1.24 **Family 38**	α-D-mannose	STSLKP**PTA**DLFTGV IPRRSW**SPAL**TIENE	247-249 601-604	A peptide D peptide	Lysosomal/ Mannosidosis
α-L-iduronidase	3.2.1.76 **Family 39**	α-L-iduronate, HS and DS	SYNFTH**LDG**YLDLL WNFETW**NEP**DHHDFD EGLRAA**SPAL**RLGGP LGRPVF**PTA**EQFRRM	114-116 181-183 213-216 496-498	proton donor 182	Lysosomal/ MPS I
α-mannosidase IA & IB	3.2.1.113 3.2.1.113 **Family 47**	1,2-α-D-mannose $Man_9(GlcNAc)_2$	GVLFHS**SPAL**QPAAD GLHDEF**LDG**QRWIED	77-80 244-246		Golgi Golgi
Hyaluronidase 2	3.2.1.35 **Family 56**	1,4- N-acetyl-β-D-glucosamine, D-glucuronate	WAMELK**PTA**PPIFTG	25-27		GPI-anchor PM

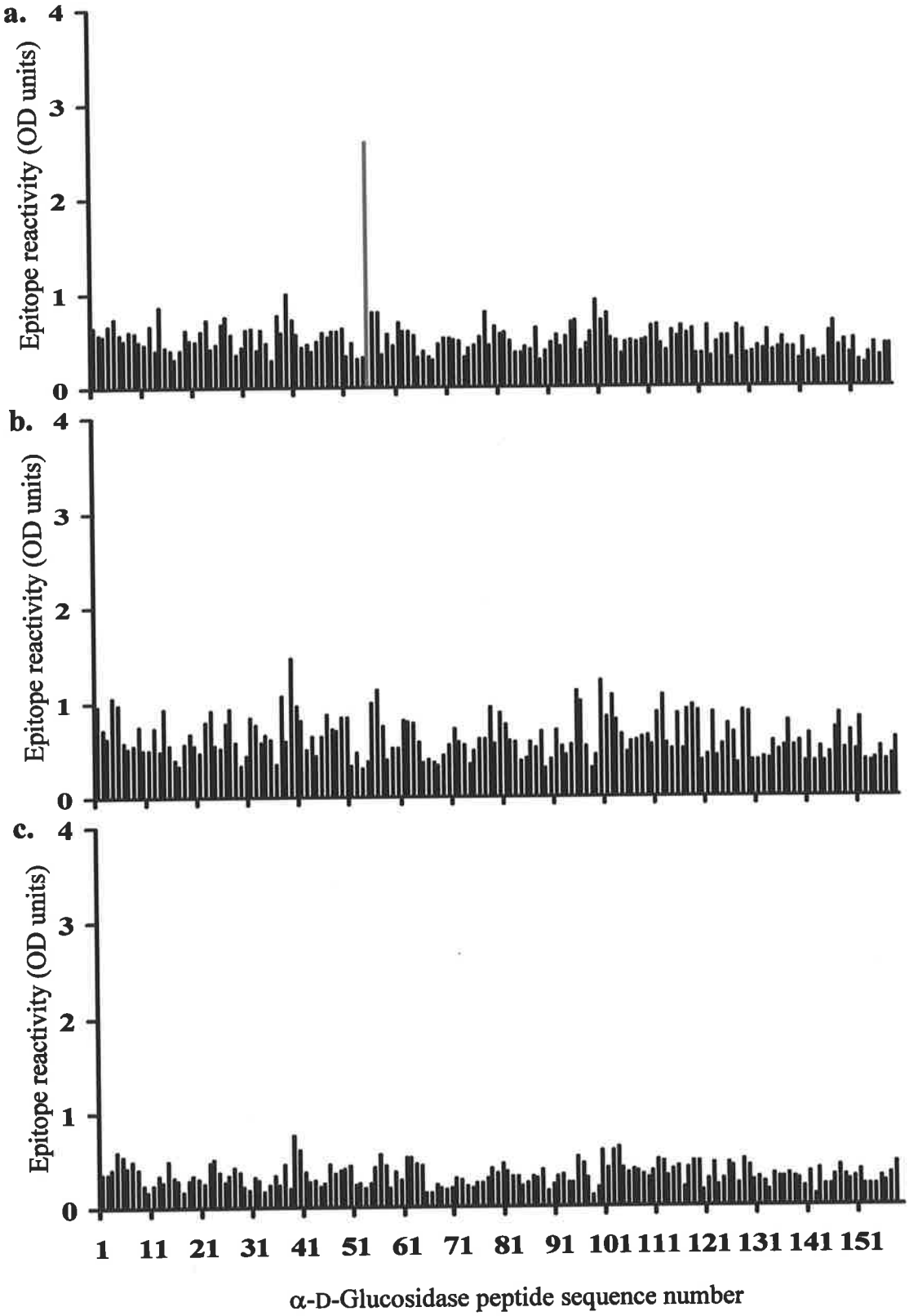

Figure 4.12: Epitope cross-reactivity of sera antibodies from α-L-iduronidase-treated MPS I patients 7 (a), 8 (b) and 9 (c) tested against α-D-glucosidase peptide sequences. The y-axis shows the level of ELISA O.D. reactivity plotted against the peptide sequence numbers (x-axis). Red bars denote high affinity antibody reactivity.

In particular, seven different glycosidases had an NEP sequence that was always located in the close vicinity of either a known proton donor or nucleophile residue (i.e. at the end of a β-sheet). While these sequences were at antigenic sites in some glycosidases, they were not specific to glycosidases, implying that the surrounding sequence or structure must have also been important components of the epitope cross-reactivity in α-D-glucosidase and α-L-iduronidase.

4.2 Discussion

There are two main catalytic mechanisms that operate in glycosyl hydrolases, involving either retention or inversion of the substrate stereochemistry at the cleavage point (McCarter and Withers, 1994). The retaining catalytic mechanism is conserved within the following glycosyl hydrolase families: 1, 2, 5, 7, 10, 11, 12, 13, 16, 17, 22, 30, 31, 32, 33, 34, 35, 39, 42 (Henrissat and Bairoch, 1996). The common catalytic mechanism in these glycosidases correlates with a conserved $(\beta/\alpha)_8$ barrel structure and a small but defined level of sequence identity, which is focused on specific residues that directly participate in the catalytic mechanism (Durand *et al.,* 1997). This structure and sequence conservation led to the investigation of the possibility of common antigenicity in glycosyl hydrolases. The linear sequence antigenicity of two distantly related glycosyl hydrolases, α-D-glucosidase (family 31) and α-L-iduronidase (family 39), was examined in detail. The selection of these glycosidases was in part due to the observation that both have generated immune responses in LSD patients undergoing treatment by ERT (Amalfitano *et al.,* 2001; Kakkis *et al.,* 2001; Aldurazyme®, 2003; Kakavanos *et al.,* 2003; Wraith, 2005).

Epitope reactivity to α-L-iduronidase in sera from MPS I patients receiving ERT was reported in Chapter 3 (Kakavanos *et al.,* 2003). Here, a similar pattern of epitope reactivity

was obtained with an anti-α-L-iduronidase polyclonal antibody where the high affinity epitopes located in the first half of α-L-iduronidase mapped to the outer α-helix elements of the $(\beta/\alpha)_8$ barrel. However, this trend was less than that previously reported for sera from immune responsive MPS I patients (Kakavanos *et al.*, 2003).

The epitope reactivity of an anti-α-D-glucosidase polyclonal antibody and a panel of monoclonal antibodies was defined against the full-length α-D-glucosidase peptide sequence. The anti-α-D-glucosidase polyclonal antibody showed low-level epitope reactivity corresponding to the peptides containing the active site residues (located at the end of β-strands forming the $(\beta/\alpha)_8$-barrel), but higher epitope reactivity to adjacent sites (located in the α-helical structures connecting the β-strands in the-barrel). This trend was similar to that seen with the anti-α-L-iduronidase polyclonal antibody on the α-L-iduronidase protein sequence, which suggested that the conserved $(\beta/\alpha)_8$ barrel contributed to the antigenicity of these two lysosomal enzymes.

The α-D-glucosidase monoclonal antibody, 43D1, showed high affinity epitope reactivity with a peptide that contained the nucleophilic acid residue of α-D-glucosidase; and three other monoclonal antibodies also reacted with an epitope located at one of the known proteolytic processing sites. This suggested that the catalytic and processing sites of α-D-glucosidase were antigenic regions of this replacement protein. Furthermore, differences in antigenic properties and responses to heat denaturation were observed for the precursor and mature forms of rh-α-D-glucosidase when using the panel of α-D-glucosidase monoclonal antibodies. This highlighted the potential effect of proteolytic processing on the accessibility of antigenic sites on α-D-glucosidase and suggested that the presence of

processed forms in the enzyme preparation could exacerbate the humoral immune response to this replacement protein. I have also shown the development of high antibody titres and high affinity epitope reactivity in rats immunised with rh-α-D-glucosidase (from transgenic rabbit milk). Several regions of high affinity epitope reactivity were identified and one of these (involving peptides 37-39) was a common antigenic region for the four immunised rats. This protein region may be important in the development and maintenance of antibody reactivity to α-D-glucosidase. In GSD II patients receiving rh-α-D-glucosidase purified from CHO-K1 culture, 83% developed an immune response to ERT (http://www.worldpompe.org/myozyme260405.html). All patients receiving rh-α-D-glucosidase from transgenic rabbit milk have developed higher than normal levels of antibody titres to the infused protein (Van den Hout *et al.,* 2004; Winkel *et al.,* 2004; Klinge *et al.,* 2005). This was not unexpected as the studies reported here showed that α-D-glucosidase was an extremely antigenic protein. Neutralising antibody reactivity was also reported in some GSD II patients receiving ERT (Amalfitano *et al.,* 2001). Therefore, further work on reducing α-D-glucosidase antigenicity by manipulating the key sites of antigenicity of this replacement protein has been proposed.

A high level of epitope reactivity was detected on both α-D-glucosidase and α-L-iduronidase, indicating a number of highly antigenic sites across both proteins. Our collaborators (Pierre Lehn, INSERM U613, Brest, France) attempted to model the positional location of the $(\beta/\alpha)_8$-barrel in α-D-glucosidase and compared this to a previous prediction for α-L-iduronidase (Durand *et al.,* 1997; 2000; Brooks *et al.,* 2001), to localise the specific epitope reactivities for these two glycosidases. The probable location of some of the secondary structure elements of the $(\beta/\alpha)_8$-barrel in α-D-glucosidase were identified, including the β2, β3, β4 (ASP518) and β7 (ASP616) sheets. In these regions of alignment

and in α-L-iduronidase, it was evident that there was generally more epitope reactivity in regions containing α-helix (outer) compared to β-sheet (inner) elements of the $(\beta/\alpha)_8$-barrel structure. The incomplete alignment of α-D-glucosidase with other glycosidases may be explained by the recent findings for the three-dimensional structure of α-D-glucosidase from *E. coli*, which showed an additional α/β domain inserted between the third β-sheet and α-helix (Lovering *et al.*, 2005).

Despite the inability to completely align the α-D-glucosidase and α-L-iduronidase sequences, we were able to show significant cross-reactivity for polyclonal antibodies that were generated to each of these two glycosidases. This cross-reactivity was mainly focused on β-sheet elements of the $(\beta/\alpha)_8$-barrel that formed the catalytic sites of the two glycosidases. However, the linear sequence at these sites of cross-reactivity only had short 3-4 amino acid lengths of sequence identity. These sequence motifs were found in some but not all glycosidases and where present corresponded mainly to β-sheet structures involved in the catalytic mechanism. For example, the NEP sequence was only detected at proton donor or nucleophile sites known to be directly involved in the active site of each glycosidase, and located at the end of β-sheet structures in the $(\beta/\alpha)_8$-barrel. Moreover, the monoclonal antibody 43D1 that reacted with the active site of α-D-glucosidase containing ASP518, cross-reacted with a similar site on α-L-iduronidase containing the E182 proton donor, and both sites contained an NEP sequence. It was likely that the cross-reactive epitopes identified, involved to some extent this short sequence identity, but also structural similarity at these critical sites.

This study demonstrated that conserved micro-structural features and regions of short sequence identity could make a contribution to common glycosidase antigenicity. For LSD

patients undergoing ERT, this suggested that even patients with null mutations (i.e. with no detectable mutant protein) may not be as immunologically naïve as first suspected. Instead, related glycosidases may contribute some background immune reactivity representative of the major antigenic sites on the missing glycosidase. Background antibody titres have been reported in animal models and in humans immediately prior to the start to the onset of therapy (Brooks *et al.,* 1997; Daly *et al.,* 2000; Kakkis *et al.,* 2001; Kakavanos *et al.,* 2003; Linthorst *et al.,* 2004). Although this reactivity could be attributed to exposure of residual mutant protein, serum antibody titres to replacement enzymes have also been detected in LSD individuals with null mutations (e.g. Brooks *et al.,* 1997). The potential read-through of premature stop codon mutations, as shown for MPS I, should be considered here as it can result in the production of small amounts of native α-L-iduronidase protein (Hein *et al.,* 2004) that may be sufficient to prime the patients' immunological system. However, background antibody reactivity has also been detected in the knockout mouse model of MPS I (Chapter 3) where no α-L-iduronidase was present. This supported the hypothesis that antibody production in response to ERT in LSD patients may be produced in response to cross-reacting antigen.

Here we have shown that glycosidases can have common antigenic sites and in some cases this involved epitopes relating to the active sites of these glycosidases. One such epitope was the first to react and last to tolerise in several ERT-treated MPS I patients (Chapter 3, Kakavanos *et al.,* 2003). Furthermore, peptide 37 (and its surrounding sequence) on α-D-glucosidase, was a common antigenic site in the four rats immunised with rh-α-D-glucosidase purified from transgenic rabbit milk. In some GSD II and Gaucher patients, neutralising antibodies (implicating active site residues) have been associated with reduced

efficacy of ERT (Amalfitano *et al.,* 2001, Brady *et al.,* 1997 respectively). This highlighted the potential importance of these epitopes in immune response to replacement proteins.

Common glycosidase antigenicity may also explain why immune response to ERT has proven to be less of an issue than initially postulated in LSD patients. I hypothesise that intravenous ERT may disturb the balance of endogenous immune reactivity (produced in response to a cross-reacting antigen), contributing to antibody reactivity in some LSD patients. This may not represent a new immune response and, over time, tolerance to these reactive antigens would be restored, as observed in MPS I patients receiving replacement enzyme (Chapter 3, Kakavanos *et al.,* 2003). It has been proposed that these common glycosidase epitopes may be used to induce tolerance in LSD patients with a glycosidase enzyme deficiency. Other strategies to minimising antibody reactivity during ERT in LSD patients would be beneficial. It is reported here that the α-D-glucosidase catalytic site was one of the antigenic regions of this protein. Therefore, targeting the catalytic site to increase α-D-glucosidase stability and consequently reduce the antibody reactivity of this lysosomal protein was proposed and formed the basis of Chapter 5.

CHAPTER 5:

EFFECT OF D-GLUCOSE ON α-D-GLUCOSIDASE EXPRESSION, PURIFICATION AND ANTIBODY REACTIVITY

5 Introduction

Unusually large quantities of rh-α-D-glucosidase (up to 40 mg/kg) are required to treat GSD II patients by ERT when compared to other LSD (1 mg/kg) (Amalfitano *et al.,* 2001; Kishnani and Howell, 2004; Van den Hout *et al.,* 2004). This may be due to inefficient α-D-glucosidase uptake, or enzyme accessibility within the major affected organs (Raben *et al.,* 2003; Zhu *et al.,* 2004). Problems during purification of the rh-α-D-glucosidase from cell culture, have included aggregation and precipitation (Van Hove *et al.,* 1997; www.worldpompe.org./synpac.html).

ERT in GSD II patients has resulted in a high incidence of antibody production to the replacement protein, hypersensitivity reactions and evidence of neutralising antibodies in some patients (Amalfitano *et al.,* 2001; Van den Hout *et al.,* 2004; Winkel *et al.,* 2004). The high incidence of antibody reactivity against α-D-glucosidase could be a result of the high enzyme dose administered to GSD II patients or the high level of antigenicity of this lysosomal protein, as shown in Chapter 4.

This study aimed to further investigate α-D-glucosidase stability and antigenicity. In Chapter 4, the α-D-glucosidase catalytic site was identified as one of the main antigenic regions of this lysosomal protein. It was postulated that stabilising the catalytic site of α-D-glucosidase could increase the amount of protein recovered during purification and may partially mask antibody reactivity against this region. The findings have relevance to the safety and efficacy of ERT in GSD II patients.

5.1 Results

5.1.1 Differential rh-α-D-glucosidase expression in media

An initial observation of low rh-α-D-glucosidase expression from a CHO-K1 cell line cultured in α-MEM media led to the investigation of the expression of this glycosidase in different cell culture media. Very little α-D-glucosidase activity was detected in the culture supernatants when α-MEM was utilised as both growth and expression culture medium (Table 5.1). The addition of either 1.5% DMSO or foetal calf serum to the α-MEM culture medium made little or no difference to rh-α-D-glucosidase expression. An eight-fold increase in α-D-glucosidase activity was observed with Ham's F12 when used as both the growth and expression media, compared to α-MEM (Table 5.1). This increase in activity was lost when the expression medium was changed from Ham's F12 back to α-MEM. The use of Coon's DMEM as an expression medium resulted in the highest α-D-glucosidase activity, with a 25-fold increase when compared to α-MEM. However, in the presence of 1.5% DMSO the activity was diminished to levels observed with α-MEM, suggesting an inhibitory effect on α-D-glucosidase activity. The variability in α-D-glucosidase activity with different cell culture media led to an investigation of the composition of each medium. One of the obvious differences was the level of D-glucose (Table 5.2); glucose concentrations of 1.0, 1.8 and 3.1 g/L were present in α-MEM, Ham's F12 and Coon's DMEM respectively, which in turn correlated with the level of α-D-glucosidase activity detected with each culture medium.

Table 5.1 : The effect of different culture media on rh-α-D-glucosidase expression in CHO-K1 cells

rh-α-D-Glucosidase CHO Cell Culture Growing Medium/ Expression Medium	rh-α-D-Glucosidase Activity (nmol/min/mL)
α-MEM / α-MEM	0.430
α-MEM / α-MEM + 1.5 % DMSO	0.342
α-MEM / α-MEM + FCS	0.513
Ham's F12 / Ham's F12	3.867
Ham's F12 / α-MEM	0.560
Ham's F12 / Coon's DMEM	10.497
Hams F12 /Coon's DMEM +DMSO	0.853
Coon's DMEM /Coon's DMEM	11.220
Coon's DMEM / α-MEM	0.612

Table 5.2: Media constituent comparison

Media Components	Concentration (mg/L)		
	α-MEM	Ham's F12	Coon's/DMEM
INORGANIC SALTS:			
CaCl2 (anhyd.)	200	33.3	151
CuSO4-5H2O	0.0025	0.0025	0.001
Ferric Nitrate-9H2O			0.05
FeSO4-7H2O	0.83	0.863	0.4
KCl	400	223.65	342
MgSO4 (anhyd.)	97.67		58.43
MgCl2	57.22		24.83
MgCl2-6H2O		57.22	
NaCl	6800	7599	6975
NaH2PO4.H2O	140		
Na2HPO4		142	
ZnSO4-7H2O	0.86	0.863	0.07
OTHER COMPONENTS:			
D-Glucose	**1000**	**1802**	**3151**
HEPES			3574.5
Hypoxanthine-2Na Salt	4.77	4.77	2.39
Linoleic Acid	0.08	0.08	0.042
Lipoic Acid	0.2	0.21	0.105
Phenol Red	10	1.2	
Putrescine 2HCl	0.161	0.16	0.161
Thymidine	10	0.7	3.65
Sodium Pyruvate	110	110	110
AMINO ACIDS			
l-Alanine	25	8.91	8.9
L-Arginine . HCl	105	211	253
L-Ascorbic Acid	50		7.5
L-Asparagine . H2O	50	15	15
L-Aspartic acid	30	13.31	13.3
L-Cystine .2HCl	24		31.29
L-Cysteine HCl . H2O	100	35	35.12
L-Glutamic Acid	75	14.7	14.7
L-Glutamine	292	146.2	438
Glycine	50	7.5	22.5
L-Histidine	50		
L-Histidine HCl . H2O		21	41.98
L-Isoleucine	52.4	4	56.47
L-Leucine	52.4	13	65.55
L-Lysine . HCl	58	36.5	109.5
L-Methionine	15	4.5	19.48
L-Phenylalanine	32	5	37.98
L-Proline	40	34.5	34.5
L-Serine	25	10.5	31.5
L-Threonine	12	59.45	
L-Tryptophan	10	2	10.02
L-Tyrosine.2Na.2H2O	52	7.8	59.68
L-Valine	46	11.7	58.7
VITAMINS:			
Biotin	0.1	0.007	0.035
D-Ca Pantothenate	1	0.5	2.24
Choline Chloride	1	14	8.98
Folic Acid	1	1.3	2.65
i-Inositol	2	18	12.6
Niacinamide	1	0.04	2.02
Pyridoxine HCl	1	0.06	0.031
Pyrodoxal Hydrochloride			2
Riboflavin	0.1	0.04	0.219
Thiamine HCl	1	0.3	2.17
Vitamin B12	1.36	1.4	0.68

5.1.1.1 The affect of increasing D-glucose concentration on rh-α-D-glucosidase expression

The effect of increasing D-glucose concentration on rh-α-D-glucosidase activity in CHO-K1 cell culture was evaluated in both α-MEM and Coon's DMEM media (Figures 5.1a-b). Culture media without the addition of extra D-glucose were utilised as baseline controls. α-MEM and Coon's DMEM, respectively, contained 1.0 g/L and 3.1 g/L of D-glucose. The expression of rh-α-D-glucosidase in α-MEM (1.0 g/L D-glucose) resulted in an initial increase in the level of α-D-glucosidase activity in the culture medium for up to 24 hours of culture, but this was followed by a progressive loss of enzyme activity for additional culture time points (Figure 5.1). The addition of extra D-glucose to α-MEM (3.1, 4.0 and 6.0 g/L final concentrations) resulted in a concentration dependent increase in α-D-glucosidase activity over a 72-hour time course. Following the 72-hour time point a plateau in α-D-glucosidase activity was observed for the 3.1 g/L D-glucose treated culture. However, the 4.0 g/L and 6.0 g/L D-glucose treated cultures continued to increase in α-D-glucosidase activity for up to 144 hours. A comparable trend of α-D-glucosidase activity over time was seen with the 3.1, 4.0 and 6.0 g/L D-glucose in Coon's DMEM media (Figure 5.1b). The increase in α-D-glucosidase activity in these culture media was associated with a similar increase in the level of rh-α-D-glucosidase protein, with mainly precursor α-D-glucosidase and mature form, evident by immune detection (Figure 5.2).

5.1.1.2 Effect of sugar and butyric acid on rh-α-D-glucosidase, rh-α-L-iduronidase and rh-4-sulphatase expression

The specificity of the effect of D-glucose on rh-α-D-glucosidase production was examined using different saccharides or energy sources and compared to other expression systems.

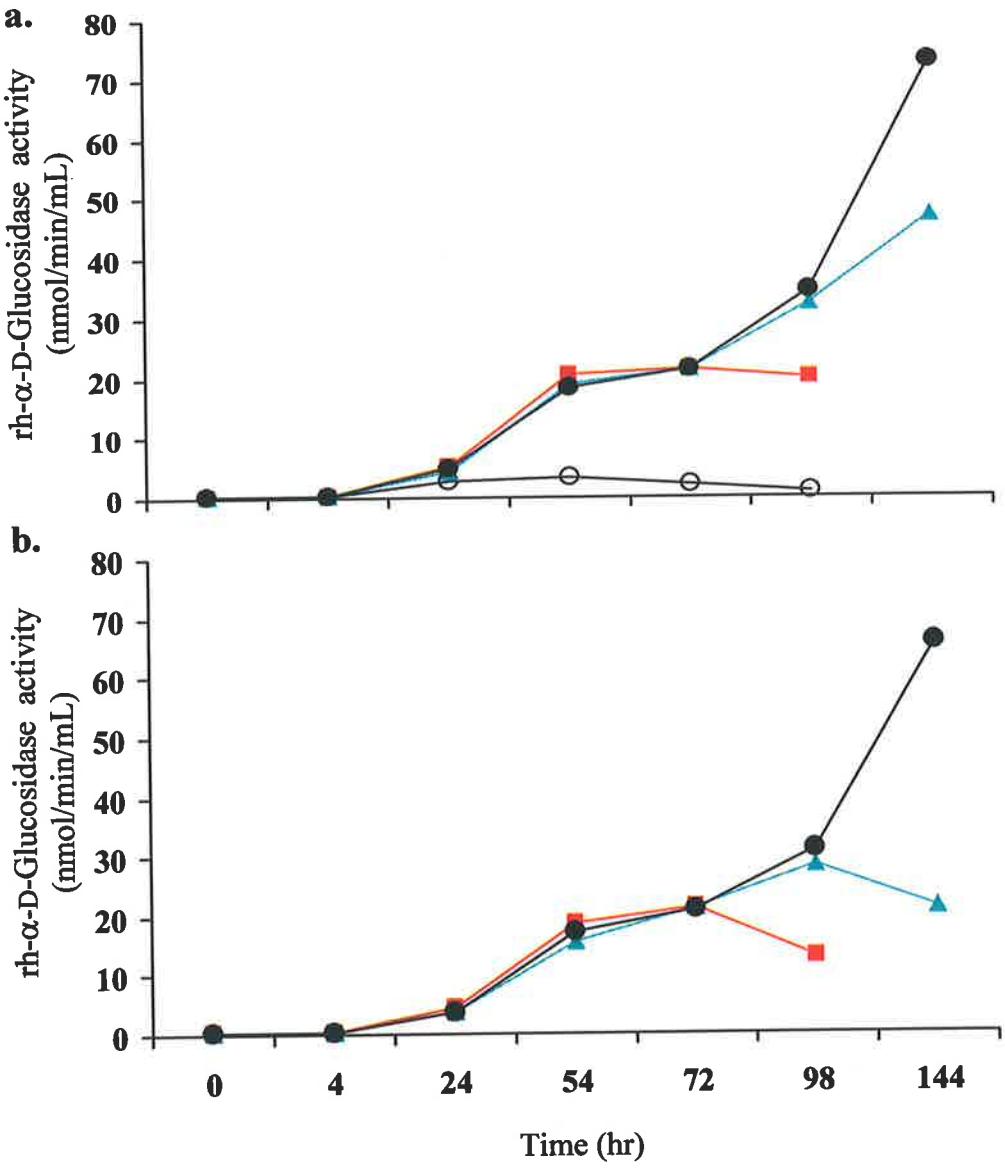

Figure 5.1: The effect of D-glucose on rh-α-D-glucosidase activity in culture over time

rh-α-D-Glucosidase activity in CHO-K1 expression cells grown in α-MEM (a) or Coon's DMEM (b) media in the presence of 1.0 g/L (O), 3.1 g/L (■), 4.0 g/L (▲) and 6.0 g/L (●) of D-glucose.

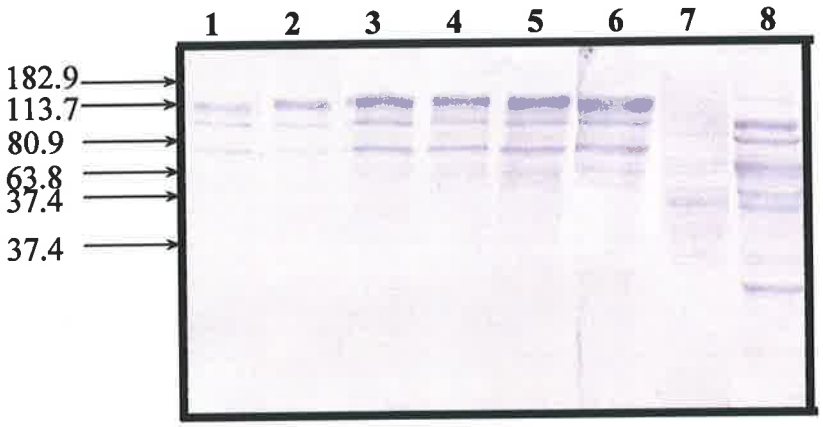

Figure 5.2: The effect of D-glucose on rh-α-D-glucosidase protein concentration

Western blot detection of rh-α-D-glucosidase protein derived from a CHO-K1 expression cell line treated with 6.0 g/L D-glucose. Lanes include media samples from the following timepoints: 4, 24, 54, 72, 98 and 144 hours post-glucose addition (lanes 1-6 respectively), and mature α-D-glucosidase from CHO cell lysates pre and post timecourse (lanes 7 & 8, respectively).

D-Glucose, D-galactose (another monosaccharide), sucrose (a disaccharide) and butyric acid (a non-sugar energy source) were investigated for their effect on CHO-K1 cells expressing three different rh-lysosomal enzymes, α-D-glucosidase, α-L-iduronidase, which share a common catalytic mechanism and structural element, and 4-sulphatase, which is structurally different from the glycosidases. For rh-α-D-glucosidase, all of the treatment groups showed an increase in enzyme activity in each culture medium for up to 72 hours of culture (Figure 5.3a). However the level of rh-α-D-glucosidase activity was higher for D-glucose (6.0 g/L) and D-galactose (6.0 g/L) suggesting an increase in production (synthesis/secretion into the medium) for these two treatment groups (Figure 5.3a). Following this time point only the D-glucose (6.0 g/L) and D-galactose (6.0 g/L) treated cultures showed an increase in rh-α-D-glucosidase activity, whereas the other treatment groups showed a progressive decline in α-D-glucosidase activity. The reduced activity in the α-MEM, sucrose and butyric acid treated CHO-K1 cultures indicated a problem with rh-α-D-glucosidase stability, but this was less evident in the D-galactose (6.0 g/L) treated culture and not evident in the D-glucose (6.0 g/L) treated culture. This showed that there was an increase in synthesis and stability of rh-α-D-glucosidase in the presence of these two monosaccharides, with the effect for D-glucose being much greater than that observed for D-galactose.

The effect of D-glucose and D-galactose on the level of rh-α-D-glucosidase produced by CHO-K1 expression cells (Figure 5.3a) was not observed for CHO-K1 cells expressing rh-α-L-iduronidase (Figure 5.3b). Instead the addition of either D-glucose, D-galactose or sucrose resulted in lower levels of rh-α-L-iduronidase activity compared to the control medium, suggesting an inhibitory effect by the sugars (Figure 5.3b). Another energy

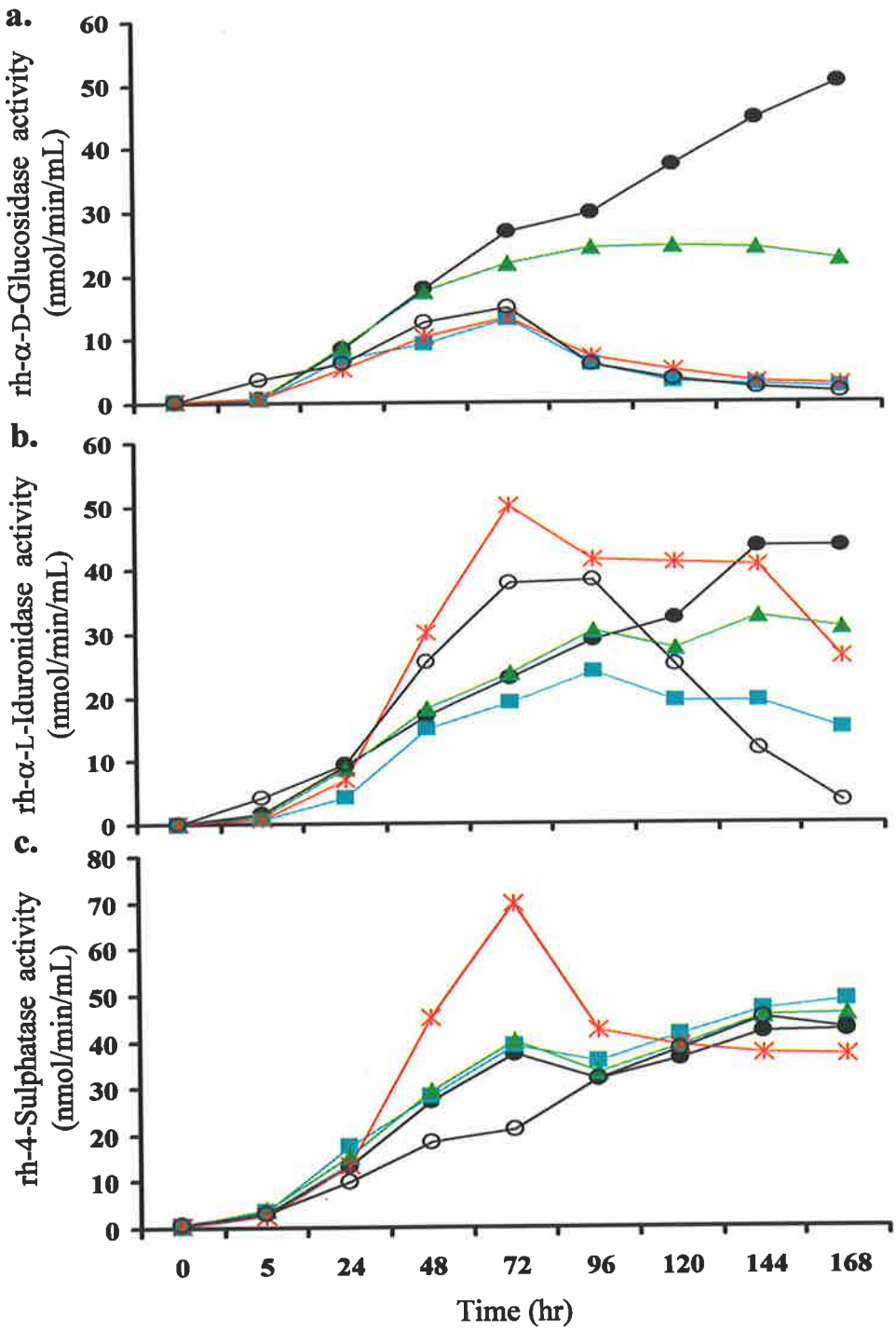

Figure 5.3: Stabilisation effect of monosaccharides on glycosidase activity

Time course of rh-α-D-glucosidase (a) rh-α-L-iduronidase (b) and rh-4-sulphatase (c) activity in Coon's DMEM media conditioned with 6.0 g/L of either D-glucose (●), D-galactose (▲), sucrose (■), with no added sugar (○) or 0.1 M butyric acid (✳).

source, butyric acid, resulted in an increase in the level of rh-α-L-iduronidase but this was not stable after 72 hours and showed a decline of α-L-iduronidase comparable to control medium (Figure 5.3b). However, there was a sugar dependent effect on rh-α-L-iduronidase stability in long-term 168-hour cultures, with 6.0 g/L D-glucose then 6.0 g/L D-galactose having the most effects (Figure 5.3b). The stability effect observed for 6.0 g/L D-glucose and 6.0 g/L D-galactose on both rh-α-D-glucosidase (Figure 5.3a) and rh-α-L-iduronidase (Figure 5.3b) was not evident for rh-4-sulphatase (Figure 5.3c). This was despite a slight increase in the production of rh-4-sulphatase with either D-glucose, or D-galactose or sucrose addition (Figure 5.3c). This slight increase in the level of rh-4-sulphatase production with sugar addition was less than that observed for butyric acid (Figure 5.3c).

5.1.2 Inhibition studies for two glycosidase enzymes

5.1.2.1 Effect of monosaccharide on purified rh-α-D-glucosidase

The monosaccharides D-glucose and D-galactose, and the disaccharide sucrose, were evaluated for inhibition of rh-α-D-glucosidase activity in a free assay system using the fluorescent substrate 4MU-α-D-glucopyranoside (Figure 5.4). Increasing concentrations of D-glucose rapidly decreased rh-α-D-glucosidase activity but this plateaued with D-glucose concentrations above 15 mg/mL. Maximum inhibition was obtained with 125 mg/mL of D-glucose with higher glucose concentrations resulting in only minimal further inhibition. D-Galactose resulted in an initial rapid decrease of rh-α-D-glucosidase activity at the lower concentrations of inhibitor but only inhibited activity to approximately one-quarter of that seen with D-glucose. In contrast, sucrose resulted in a constant slow rate of reduction of rh-α-D-glucosidase activity.

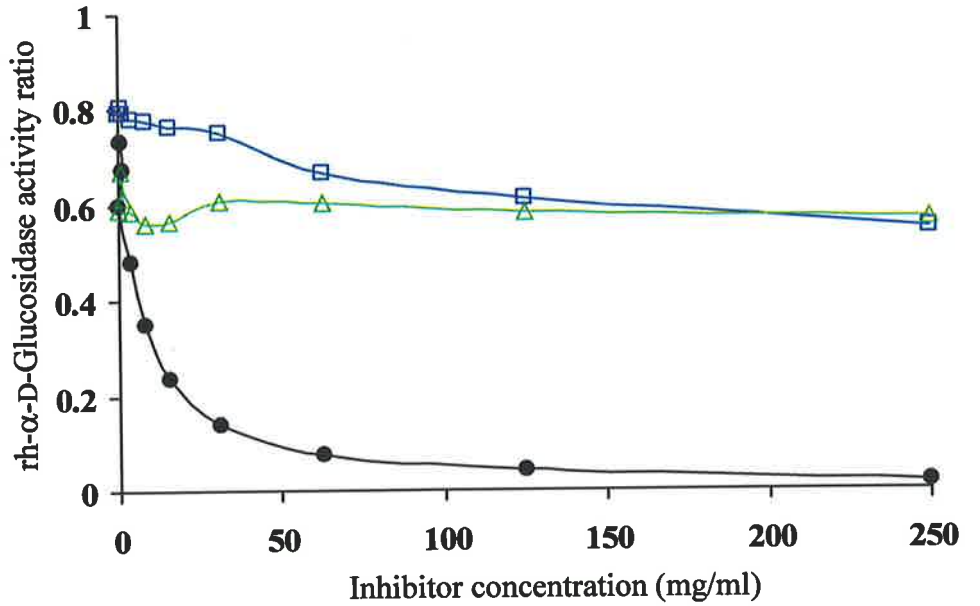

Figure 5.4: The inhibitory effect of saccharides on rh-α-D-glucosidase activity

rh-α-D-Glucosidase activity (x-axis) in the presence of increasing concentrations of either D-glucose (●), D-galactose (△) and sucrose (□).

5.1.2.2 Comparison of rh-α-D-glucosidase inhibition by D-glucose

In the presence of increasing D-glucose inhibitor concentrations rh-α-D-glucosidase purified from CHO-K1 cell culture medium and from transgenic rabbit milk resulted in a reduction in maximum velocity for the hydrolysis of the 4MU-α-D-glucopyranoside substrate (Figure 5.5). The K_m and relative V_{max} values were deduced from Lineweaver-Burk plots and respectively included 6.7 mM and 12.5 nmol/min for rh-α-D-glucosidase from transgenic rabbit milk and 2.4 mM and 5 nmol/min for rh-α-D-glucosidase from CHO-K1 culture (Figures 5.5 a-b). rh-α-D-Glucosidase purified from CHO-K1 culture had a third of the K_m compared to enzyme purified from transgenic rabbit milk and just under half the maximum velocity. In the presence of D-glucose, the K_m for rh-α-D-glucosidase increased in proportion to the concentration of D-glucose, but the V_{max} remained constant. This indicated that D-glucose was acting as a competitive inhibitor for α-D-glucosidase (Figure 5.5). Plots of slope (from the Lineweaver-Burk plots) versus glucose inhibitor concentration were used to determine the K_i values (Figure 5.6); glucose had similar K_i values for both sources of α-D-glucosidase (respectively 33 mM and 45 mM).

5.1.2.3 Inhibition of rh-α-L-iduronidase by D-glucose

Lineweaver-Burk plots demonstrated a change in both the V_{max} (47.6 nmol/min) and K_m (0.14 mM) values for rh-α-L-iduronidase in the presence of D-glucose, using the 4MU-iduronide substrate, indicating that it was acting as an uncompetitive inhibitor (Figure 5.7b). The difference in inhibition observed for rh-α-D-glucosidase and rh-α-L-iduronidase indicated that D-glucose was binding directly to the active site of rh-α-D-glucosidase, but not for rh-α-L-iduronidase. It should also be noted that the Lineweaver-Burk plots for

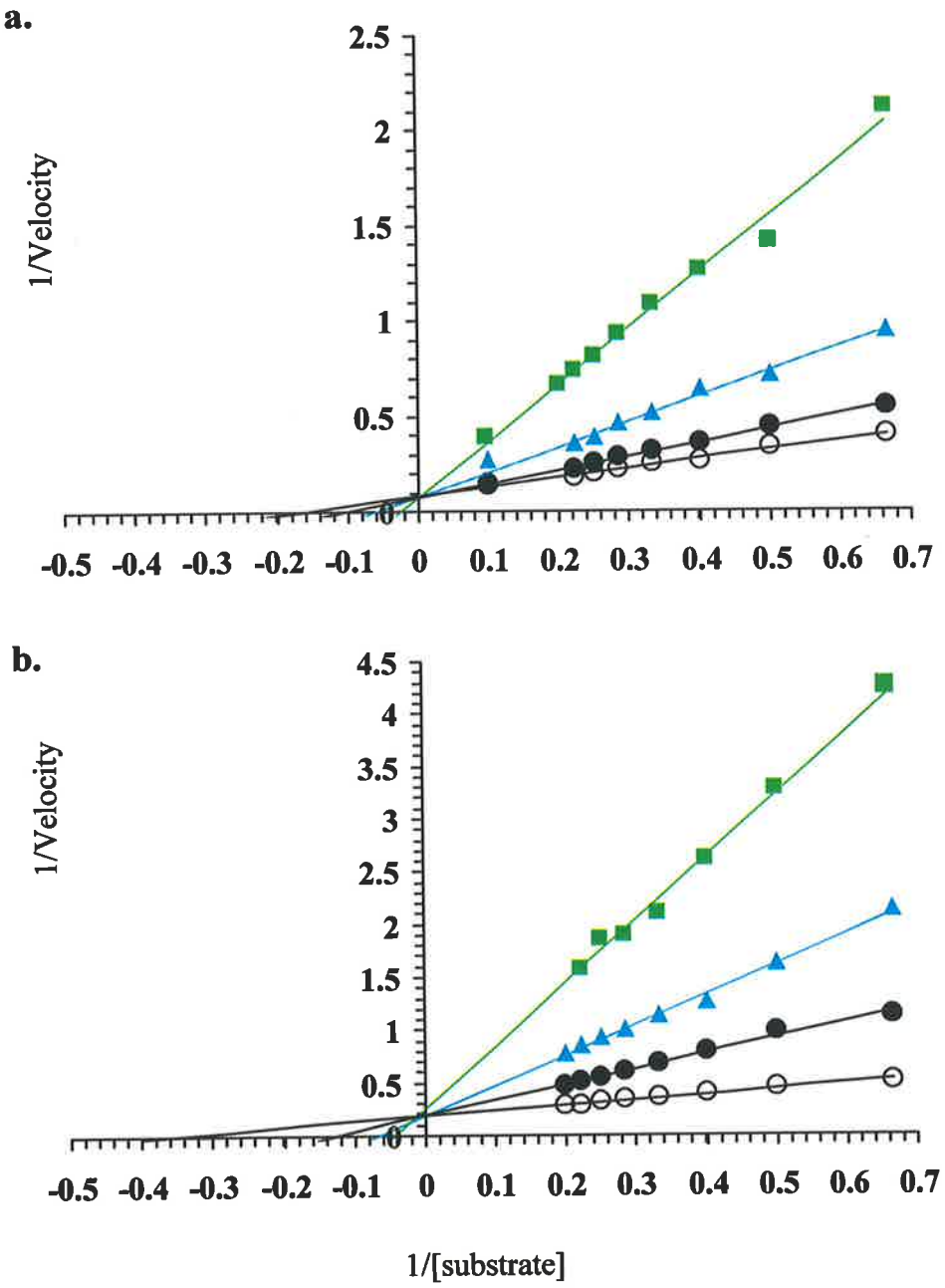

Figure 5.5: Competitive inhibition of rh-α-D-glucosidase by D-glucose

Lineweaver-Burk plots of rh-α-D-glucosidase from transgenic rabbit milk (a) and CHO-K1 culture medium (b) in the presence of the inhibitor D-glucose at concentrations of 0 (○), 0.033 (●), 0.14 (▲) and 0.28 (■) mmol/L.

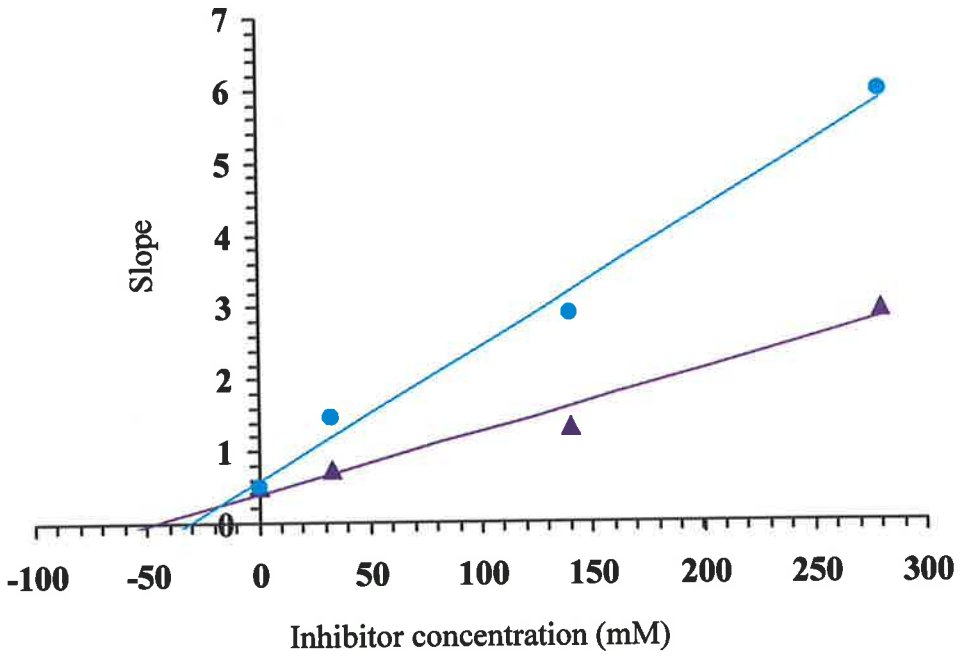

Figure 5.6: Inhibition constants of D-glucose for rh-α-D-glucosidase

Plots of slope (from the Lineweaver-Burk plots; y-axis) versus D-glucose inhibitor concentration (x-axis) for rh-α-D-glucosidase purified from transgenic rabbit milk (▲) and from CHO-K1 culture supernatant (●). The inhibition constant K_i was determined as the point of intersection on the x-axis.

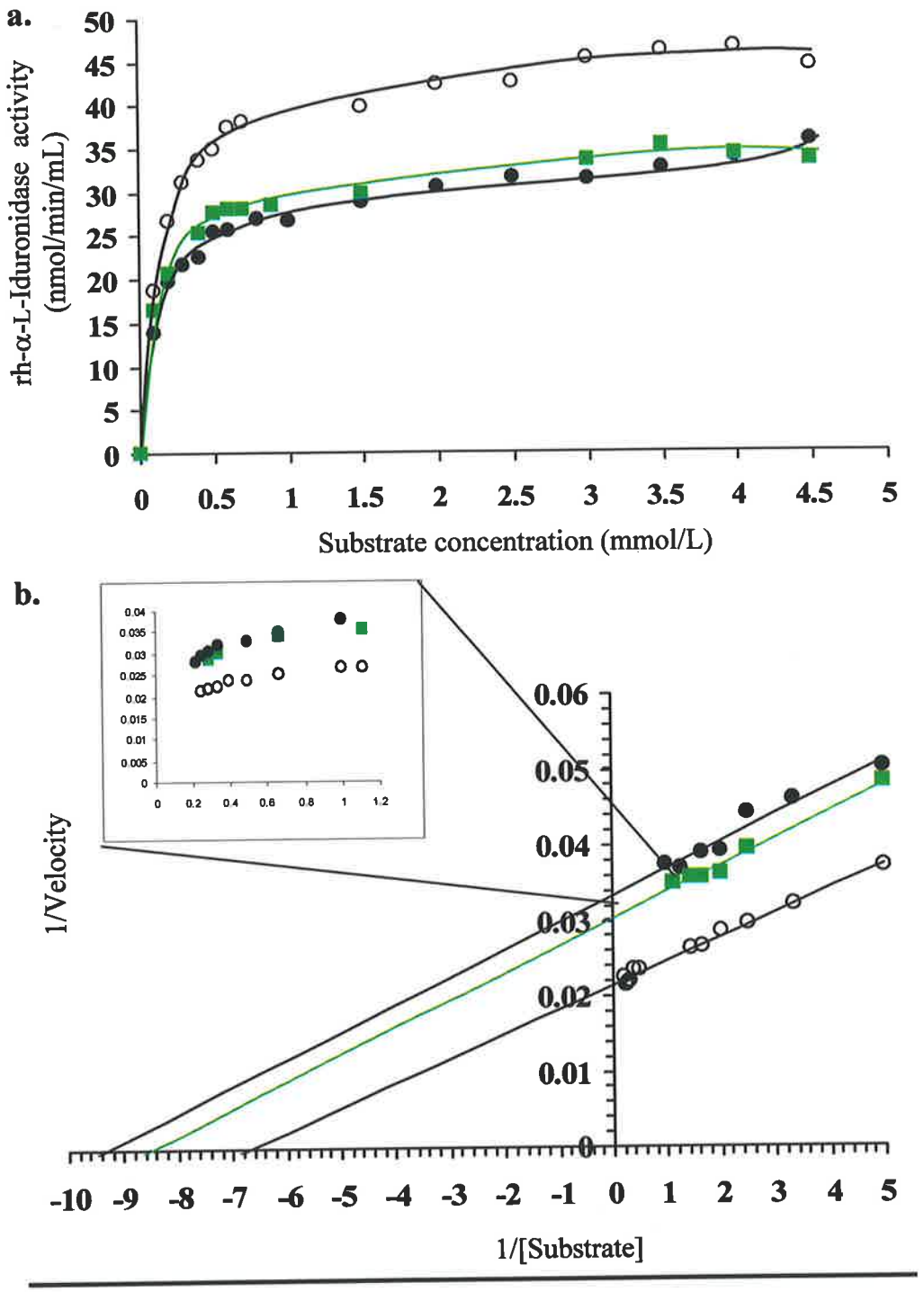

Figure 5.7: Uncompetitive inhibition of rh-α-L-iduronidase by D-glucose

Velocity (a) and Lineweaver-Burk plots (b) of rh-α-L-iduronidase in the presence of the inhibitor D-glucose at 0 (○), 0.033 (●) and 0.28 (■) mmol/L.

rh-α-L-iduronidase in the presence of D-glucose curved towards the x-axis at the higher substrate concentrations (Figure 5.7b) suggesting an activation effect.

5.1.3 Improved yield of rh-α-D-glucosidase from CHO-K1 cell cultures treated with D-glucose

Purification of rh-α-D-glucosidase from CHO-K1 cell culture using a previously reported method (Oude Elferink *et al.,* 1984; Fuller *et al.,* 1995) resulted in precipitate formation during concentration and gel filtration, consequently leading to purification yields as low as 10 percent (Table 5.3). As a result of the increased production and stabilisation of rh-α-D-glucosidase in CHO-K1 cell cultures supplemented with D-glucose, we also investigated D-glucose for its ability to improve the efficacy of rh-α-D-glucosidase purification. 125 mg/mL of D-glucose almost completely inhibited rh-α-D-glucosidase activity (Figure 5.4). The addition of 125 mg/mL of D-glucose, to the concanavalin A column eluate prior to concentration and loading onto the Sephadex G-100 gel filtration column, resulted in the elimination of the previously visible precipitate and improved the yield (Table 5.4). Yields were also improved by using A-P buffer in the culture medium (Table 5.4) as previously reported by Van Hove and colleagues (1997). Recirculation of the methyl-glucopyranoside elution buffer over the concanavalin A column maximised rh-α-D-glucosidase recovery from the column. The loss of rh-α-D-glucosidase during concentration and gel filtration chromatography was reduced by the addition of 125 mg/ml D-glucose and 0.1% Tween 20 to the concanavalin A eluate. This modified purification protocol resulted in a 3.5 fold increase in final yield when compared to the previously reported method (Tables 5.3). Immune analysis of purified rh-α-D-glucosidase indicated the presence of predominantly

Table 5.3: rh-α-D-Glucosidase purification yields using conventional methods

Sample	Activity (nmol/min/mL)	α-D-GAA (μg/mL)	Volume (mL)	Total α-D-GAA (μg)	Yield (%)
Media	51.04	85.07	55	4678.85	100
Con A Flowthrough	6.5	10.83	54	584.98	12.5
Con A Eluate + Conc	450	750	3	2250	48.1
Sephadex Pool	29.85	49.75	18	895.5	19.1
Concentrated Pool	98.2	163.67	2.8	458.28	9.8

Con A = Concanavalin A

α-D-GAA = α-D-Glucosidase

Table 5.4: Improvised purification of rh-α-D-glucosidase

Sample	Activity (nmol/min/mL)	α-D-GAA (μg/mL)	Volume (mL)	Total α-D-GAA (μg)	Yield (%)
Media	6.69	11.2	1000	11200	100
Media + AP buffer	4.5	7.6	2000	15200	137.7
Con A Flowthrough	0.17	0.28	1970	551.6	4.9
Recirculated Con A Eluate	319.9	533.2	9.5	5065.4	45.2
Con A Eluate + Glucose	225.9	376.4	10	5332	47.6
Concentrated Eluate	1050.9	1751.66	2	3503.32	31.2
Sephadex Eluate	82.5	138	30	4140	36.9
Concentrated Sephadex Eluate	704.3	1173.8	3.5	4108.3	36.7

Con A = Concanavalin A

α-D-GAA = α-D-Glucosidase

precursor rh-α-D-glucosidase (110 kDa) with minor quantities of the 95 kDa and 76 kDa mature forms (data not shown).

5.1.4 Effect of D-glucose on α-D-glucosidase activity in GSD II patient skin fibroblasts

The ability of D-glucose to stabilise mutant α-D-glucosidase was investigated in GSD II patient skin fibroblasts. Either BME or BME supplemented with D-glucose (6.0 g/L) was added to confluent GSD II skin fibroblasts and incubated for a 72-hour period. An increase in the amount of α-D-glucosidase protein was observed for normal and adult-onset GSD II patient skin fibroblasts in the presence of 6.0 g/L D-glucose (Figure 5.8), when compared to the control BME medium. The increase in α-D-glucosidase protein corresponded to a similar increase in α-D-glucosidase activity (Table 5.5). In contrast, only two of the six infantile-onset GSD II patient cell lines showed a detectable increase in α-D-glucosidase protein with D-glucose treatment (Figure 5.8), but this protein had no detectable α-D-glucosidase activity (Table 5.5). In all fibroblasts tested (GSD II and normal control), four other lysosomal markers β-glucuronidase, Saposin C, LAMP 1 and iduronate-2-sulphatase, showed either no change or a slight reduction in protein after D-glucose treatment (data not shown).

5.1.5 Differential antigenicity of rh-α-D-glucosidase from transgenic rabbit milk and a CHO-K1 cell line

5.1.5.1 Antigenicity of rh-α-D-glucosidase in enzyme-treated animals

The antigenicity of rh-α-D-glucosidase purified from either transgenic rabbit milk or CHO-K1 culture supernatant (in the presence of glucose, see section 2.6.2) was evaluated in

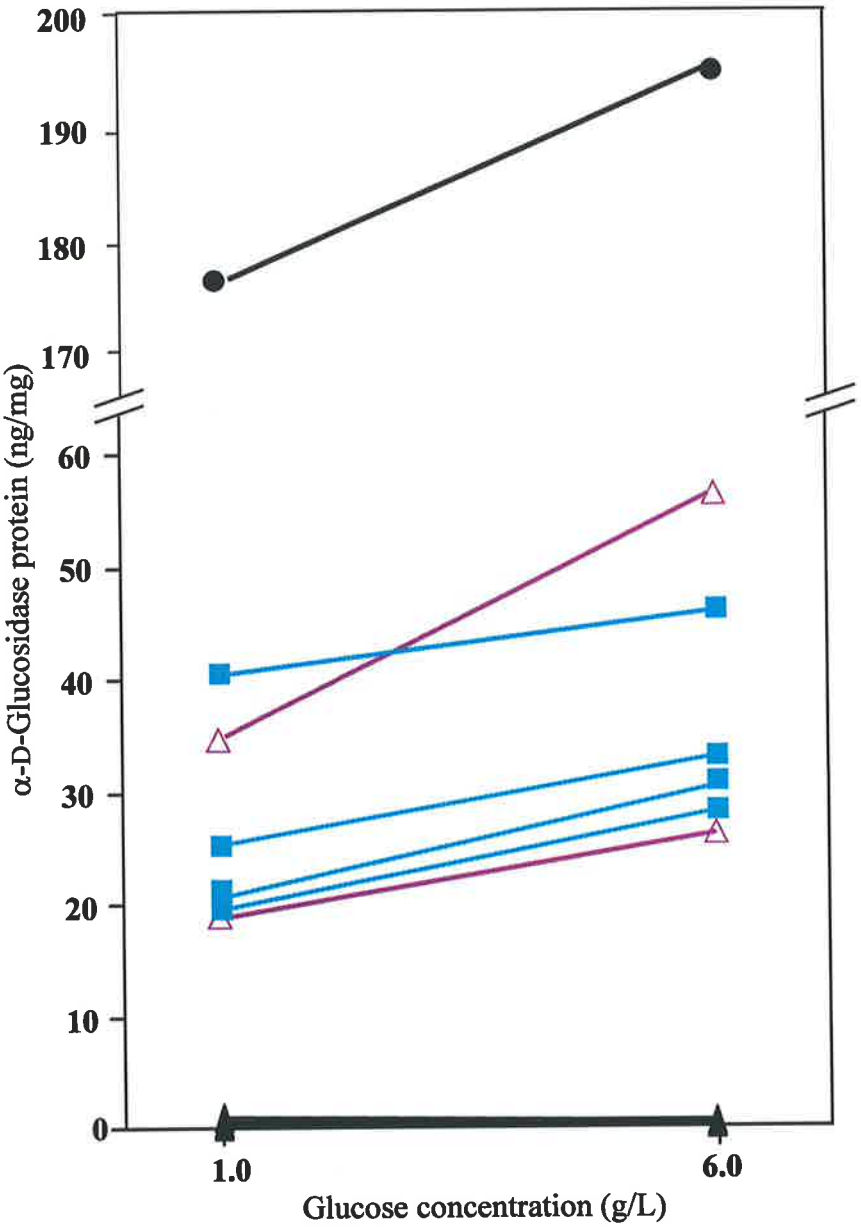

Figure 5.8: α-D-Glucosidase protein levels in GSD II patient skin fibroblasts treated with D-glucose

The effect of D-glucose on α-glucosidase protein levels of normal control (●) and GSD II patient skin fibroblast cell lines from either infantile-onset patients with negligible levels of α-D-glucosidase protein (▲), infantile-onset patients with mutant α-D-glucosidase protein (△), or adult-onset patients (■). Fibroblast cell lines were cultured either in the presence or absence of D-glucose for 72 hours before determining the level of α-D-glucosidase protein.

Table 5.5 : α-D-Glucosidase activity levels in GSD II patient skin fibroblast treated with D-glucose

Skin Fibroblast Phenotype	α-D-Glucosidase Activity (nmol/h/mg)	
	1.0mg/mL Glucose	6.0 mg/mL Glucose
Normal control	101.79	120.06
Infantile-onset 1	1.22	0.40
Infantile-onset 2	0.29	0.38
Infantile-onset 3	0.22	0.23
Infantile-onset 4	0.25	0.23
Infantile-onset 5	0.24	0.25
Infantile-onset 6	0.25	0.24
Adult-onset 1	40.63	48.85
Adult-onset 2	21.07	32.9
Adult-onset 3	13.64	18.15
Adult-onset 4	13.03	17.38

C57Bl/6 mice and Sprague Dawley rats. In both species, those treated with rh-α-D-glucosidase purified from transgenic rabbit milk developed high antibody titres (51,200 to 204,800 in mice and 818,200 to 3,276,800 in rats) (Figures 5.9a and 4.7 respectively). In one mouse treated with rh-α-D-glucosidase from rabbit milk, only a transient elevation in antibody titre was seen at week 2, which was followed by a rapid decrease in titre for subsequent time points (Figure 5.9a). The animals administered with rh-α-D-glucosidase purified from the CHO-K1 cell line had titre levels within the control range (200 to 1600 in mice (Figure 5.9b) and 100 to 800 in rats (data not shown)). This indicated a difference in α-D-glucosidase antigenicity for the two enzyme sources.

5.1.5.2 Epitope reactivity of serum antibodies from mice treated with rh-α-D-glucosidase

Three of five mice treated with rh-α-D-glucosidase from transgenic rabbit milk displayed high-affinity epitope reactivity with multiple peptides across the α-D-glucosidase protein sequence (mice 3-5; Figures 5.10a-c), consistent with the observed high antibody titres. For some of these high-affinity epitopes the positional location of antibody reactivity was partially coincident for the different mice sera, but the precise linear sequence epitopes varied between the mice. The region consisting of peptides 60-62 and peptides 127-130 on the α-D-glucosidase protein were common to all mice that showed high affinity epitope reactivity (3 of 5; Figure 5.10a-c). The region of peptides 19-22, peptides 27-28, peptide 45 and peptide 103 were common to two of the three mice that showed high affinity epitope reactivity. The fourth mouse treated with rh-α-D-glucosidase from transgenic rabbits milk showed low affinity epitope reactivity with several peptides across the α-D-glucosidase protein suggesting that the majority of antibody reactivity was to conformational epitopes (Figure 5.10d). The fifth mouse only showed little or low affinity epitope reactivity across

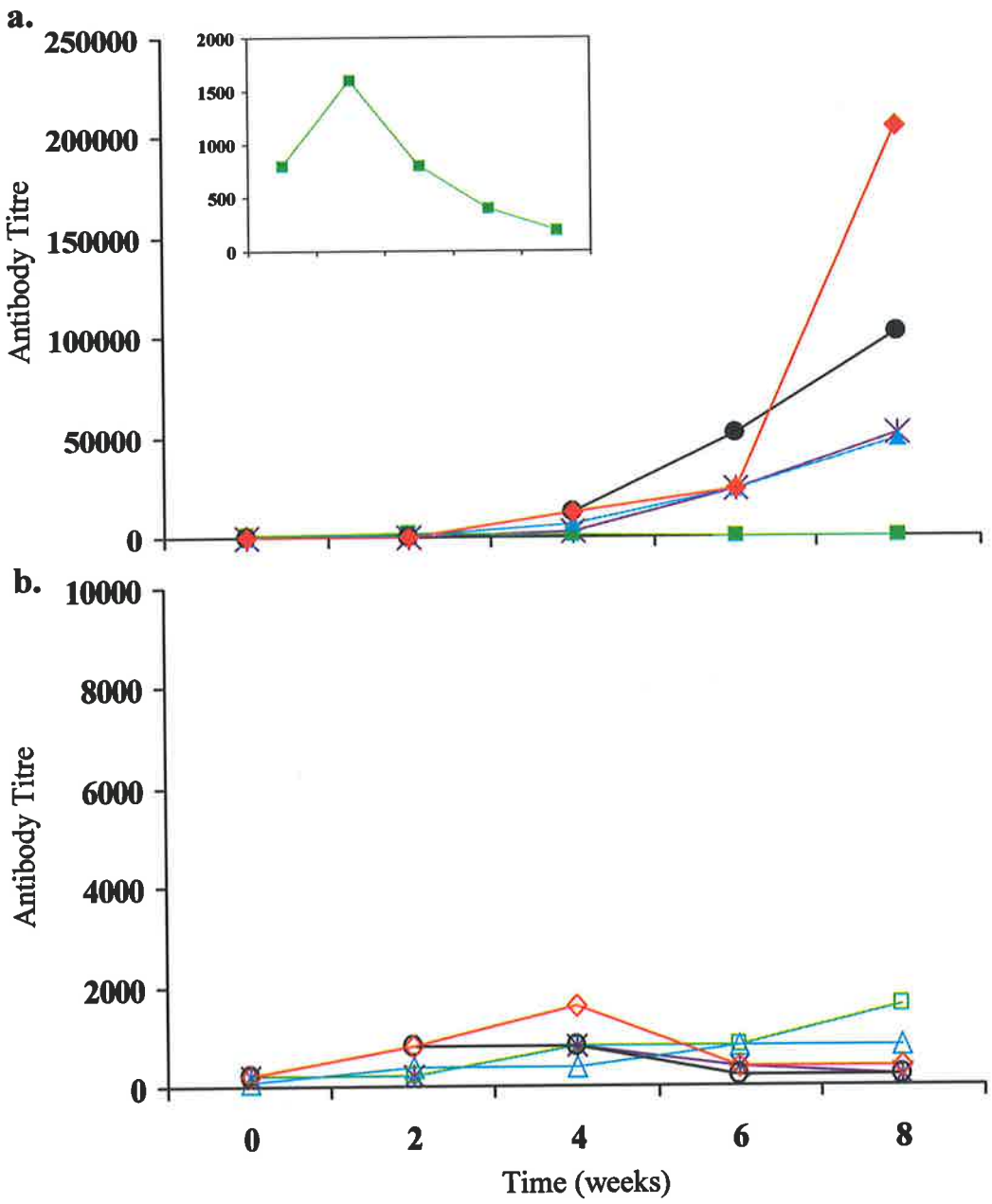

Figure 5.9 : Development of antibody titres to rh-α-D-glucosidase in ERT-treated C57Bl/6 mice

Serum antibody titres to α-D-glucosidase over time in mice treated with rh-α-D-glucosidase purified from either transgenic rabbit milk (a) or a CHO-K1 expression cell line (b). The inset in (a) shows the transient increase in antibody titre to the infused enzyme in one mouse. The different symbols represent different animals within each treatment group.

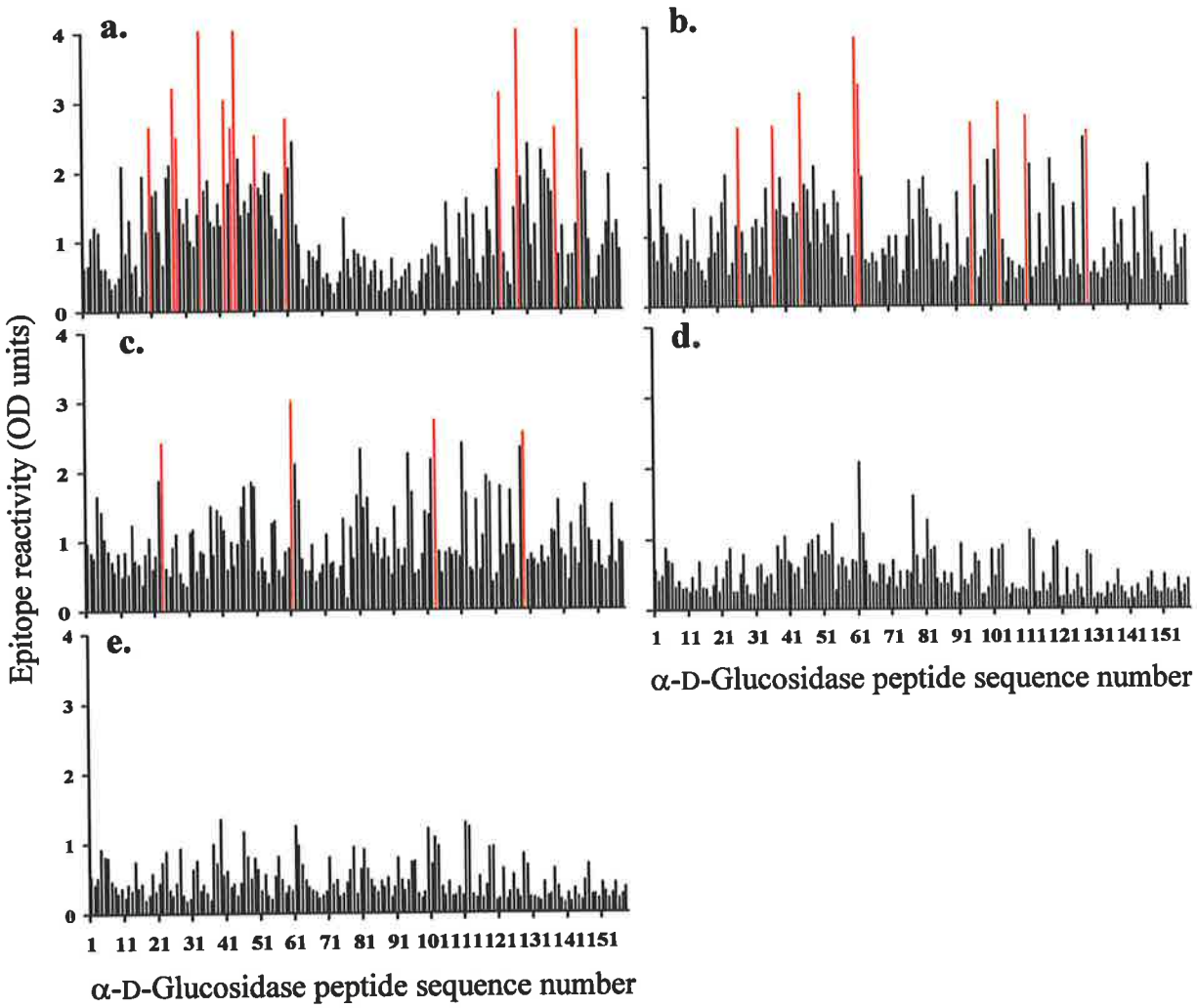

Figure 5.10: Epitope reactivity of serum antibodies from mice infused with rh-α-D-glucosidase from transgenic rabbit milk

Epitope reactivity in ERT treated mice 1-5 (a-e respectively) was expressed as ELISA OD units (y-axis) and was shown for individual peptide sequences (x-axis). Red bars denote high affinity antibody reactivity.

the α-D-glucosidase protein sequence (Figure 5.10e), which was consistent with the transient increase in antibody titre seen in this mouse (Figure 5.9a). Sera from mice treated with rh-α-D-glucosidase from CHO-K1 culture displayed little or low epitope reactivity against the α-D-glucosidase protein (Figure 5.11), which correlated with the low antibody titres observed in these mice (Figure 5.9b).

5.1.6 Reduction of antibody reactivity to rh-α-D-glucosidase in the presence of D-glucose

The difference in antibody response between rh-α-D-glucosidase purified from transgenic rabbits milk compared to that of CHO-K1 culture (using D-glucose in the purification protocol) led to the investigation of the ability of D-glucose to reduce antibody reactivity to α-D-glucosidase. rh-α-D-Glucosidase purified from transgenic rabbit milk, CHO-K1 culture medium and CHO-K1 cell lysate, showed reduced antibody reactivity with the monoclonal antibody 43D1 (epitope against the active site) in the presence of increasing D-glucose concentrations compared to the 'no inhibitor' control (Figure 5.12).

To determine whether the effect of D-glucose was specific to rh-α-D-glucosidase, two other lysosomal enzymes, rh-α-L-iduronidase and rh-4-sulphatase, were also evaluated: there was no reduction in antibody reactivity with the respective polyclonal antibody (raised against each enzyme) in the presence of 6 g/L of but there was some inhibition observed at a concentration of 50 g/L D-glucose for both lysosomal enzymes (Figure 5.13). The effect at high D-glucose concentrations may be due to the high viscosity of the D-glucose solution as it was observed for all the enzymes.

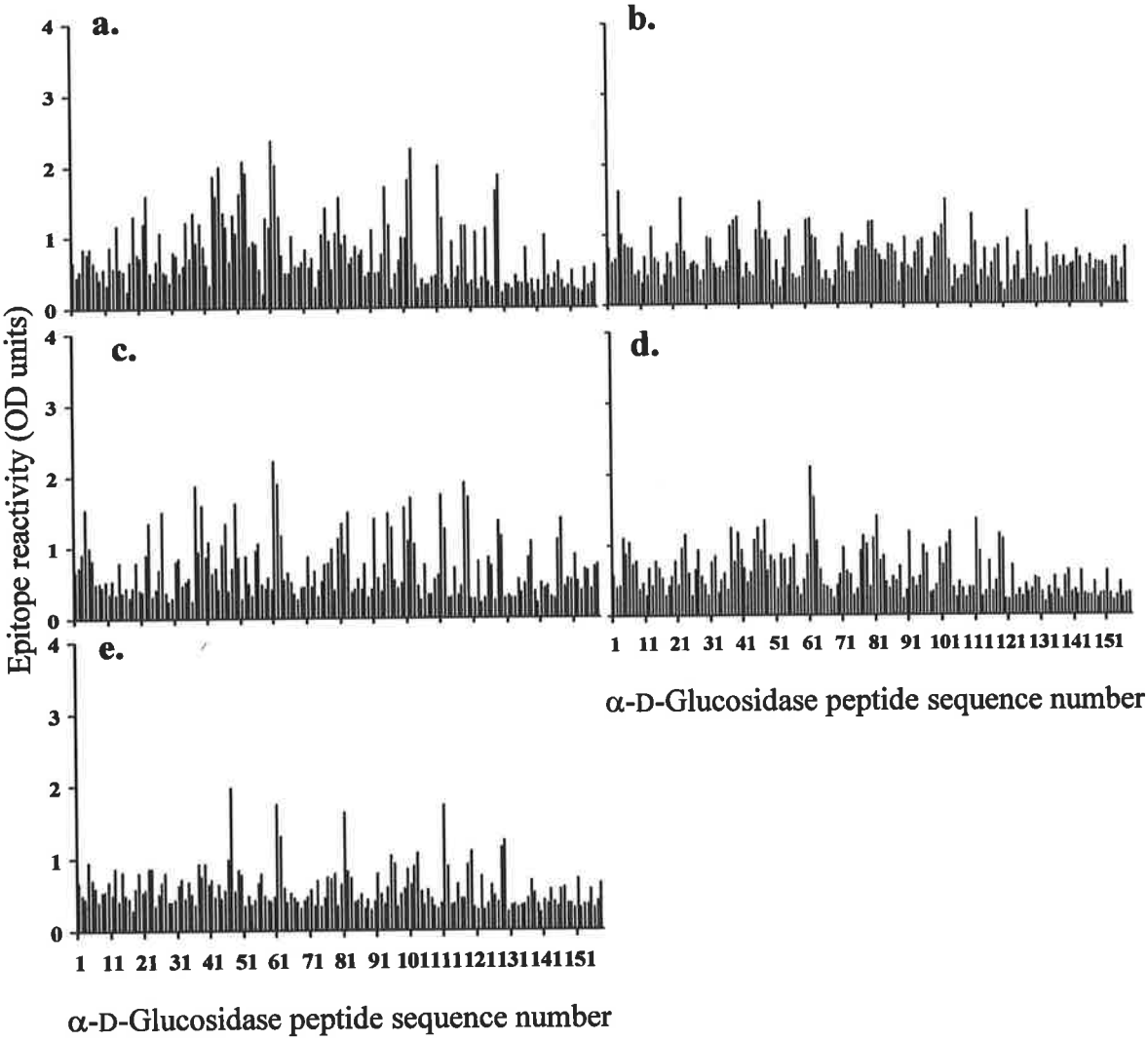

Figure 5.11: Epitope reactivity of serum antibodies from mice infused with rh-α-D-glucosidase purified from CHO-K1 culture medium

Epitope reactivity in mice 6-10 (a-e respectively) treated with rh-α-D-glucosidase from CHO-K1 culture medium was expressed as ELISA OD units (y-axis) and was shown for individual peptide pins (x-axis). Red bars denote high affinity antibody reactivity.

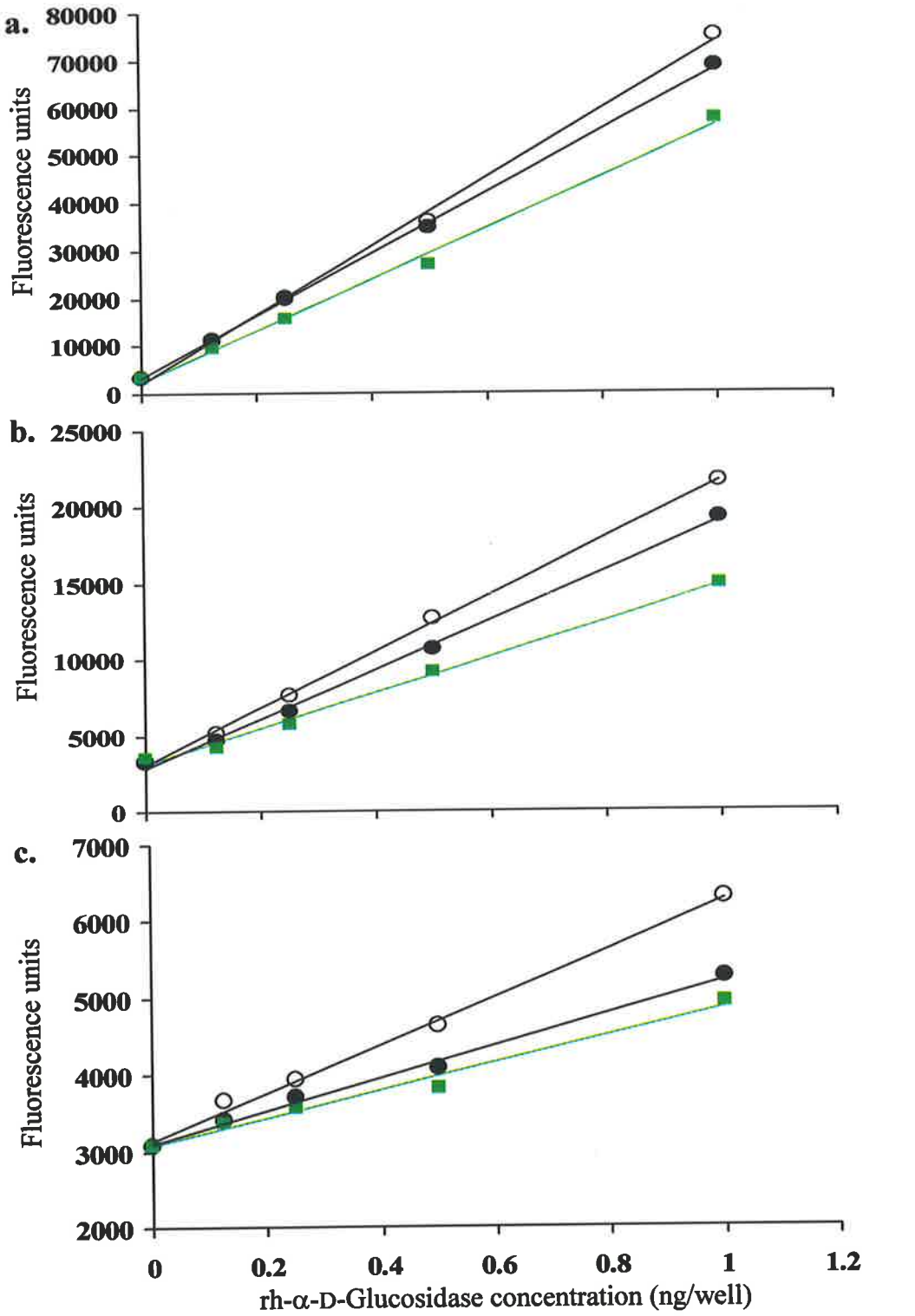

Figure 5.12: Reduced rh-α-D-glucosidase antibody reactivity in the presence of D-glucose

Antibody reactivity against the α-D-glucosidase active site in the presence of the inhibitor D-glucose at 0 (○), 0.033 (●) and 0.28 (■) mmol/L. Purified preparations of rh-α-D-glucosidase from transgenic rabbit milk (a) CHO-K1 cell lysate containing the mature form of α-D-glucosidase (b), and CHO-K1 culture supernatant (c), were captured and detected with a europium-labelled monoclonal antibody 43D1 that recognises the α-D-glucosidase active site.

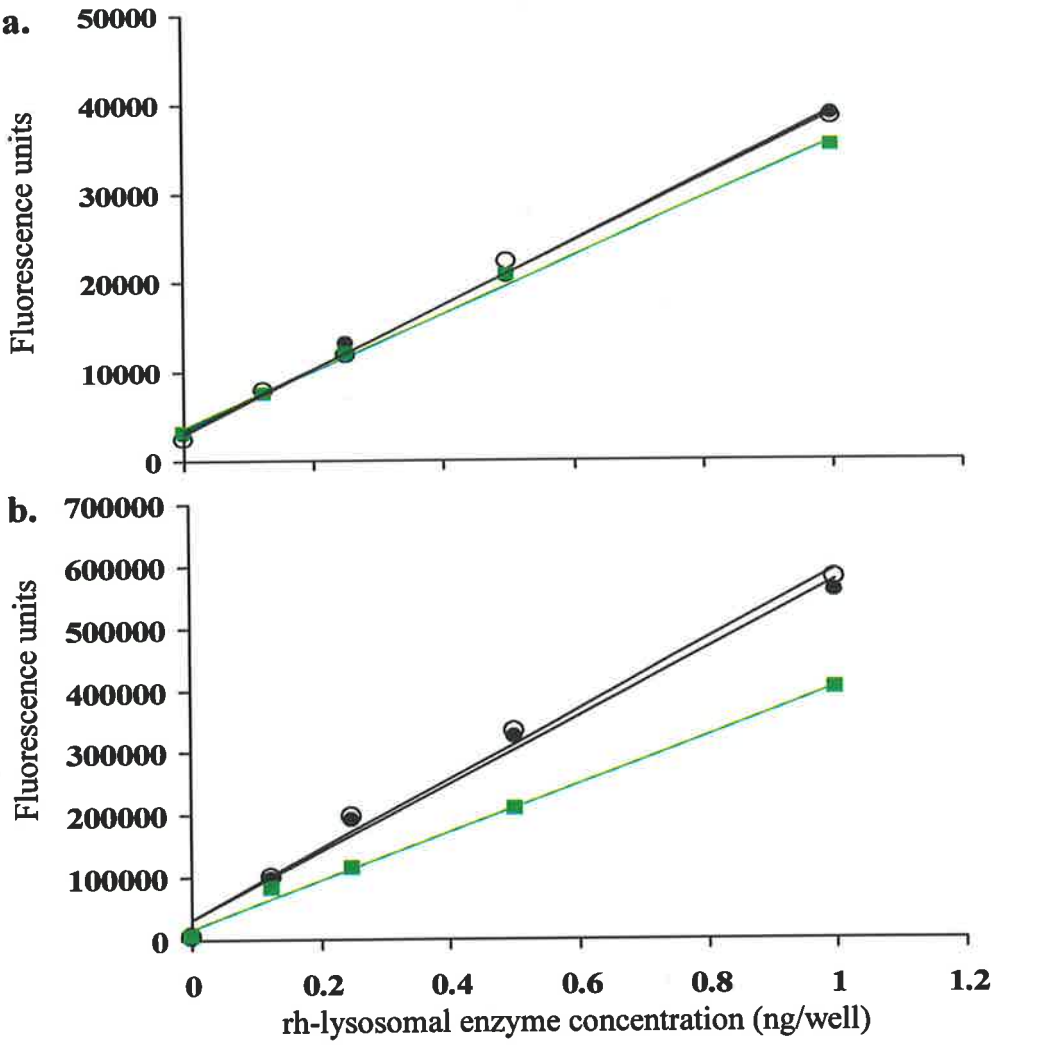

Figure 5.13: Effect of D-glucose on antibody reactivity against rh-4-sulphatase and rh-α-L-iduronidase

Antibody reactivity against rh-α-L-iduronidase (a) and rh-4-sulphatase (b) in the presence of the inhibitor D-glucose at 0 (○), 0.033 (●) and 0.28 (■) mmol/L.

5.2 Discussion

Relative to other LSD types effective ERT in GSD II patients requires large doses of rh-α-D-glucosidase. The purification of rh-α-D-glucosidase from CHO-K1 cell culture medium has addressed this need, but there have been problems with aggregation and precipitation of the purified protein (Van Hove *et al.,* 1997; www.worldpompe.org./synpac.html). Furthermore, a potential complication to ERT in GSD II patients is the high incidence of antibody development to the rh-α-D-glucosidase replacement protein (Amalfitano *et al.,* 2001; Kishnani and Howell, 2004; Van den Hout *et al.,* 2004; Winkel *et al.,* 2004; Klinge *et al.,* 2005) and the potential to develop neutralising antibodies (Amalfitano *et al.,* 2001). In addition to the inherent antigenicity of the rh-α-D-glucosidase protein, aggregate formation during purification of this lysosomal protein may also be contributing to the high incidence of antibody development in response to ERT. In Chapter 4, the catalytic site was identified as a major antigenic site in rh-α-D-glucosidase. This led to the investigation of ways to stabilise the α-D-glucosidase catalytic site using a product of enzyme catalysis, with the aim of increasing the amount of rh-α-D-glucosidase protein and potentially reducing antigenicity of the active site.

Tissue culture experiments indicated differences in the level of secreted rh-α-D-glucosidase with different expression media. The media utilised had different levels of D-glucose and this correlated with the level of rh-α-D-glucosidase expression. D-Glucose is the product of α-D-glucosidase hydrolysis and has the capacity to inhibit the enzyme; it was therefore an attractive candidate to interact with and stabilise the α-D-glucosidase catalytic site. This led to the investigation of the effect of D-glucose on rh-α-D-glucosidase production in an over-expressing CHO-K1 cell line (Fuller *et al.,* 1995).

D-Glucose was shown to be a competitive inhibitor of α-D-glucosidase, specifically interacting with the catalytic site. At a concentration of 6.0 g/L (33 mM), D-glucose was able to significantly increase the production (synthesis/secretion) of rh-α-D-glucosidase in to CHO-K1 expression medium. The concentration of D-glucose at which this biological effect was observed, was similar to the K_i for rh-α-D-glucosidase (45 mM) consistent with the D-glucose effect being mediated by binding to the active site of α-D-glucosidase. In the presence of D-glucose or D-galactose, α-D-glucosidase activity in the culture medium from CHO-K1 cells was maintained for greater than 72 hours after treatment, but in the absence of these monosaccharides, a progressive drop in rh-α-D-glucosidase activity was observed following this time point. Therefore, D-glucose treatment not only increased the amount of rh-α-D-glucosidase produced but also provided a stabilising effect in the culture medium. The increased production of rh-α-D-glucosidase may have resulted from stabilising α-D-glucosidase in the biosynthetic compartment of the CHO-K1 expression cells. This effect was not just provision of an additional energy source, as butyric acid did not increase rh-α-D-glucosidase production.

A previous study reported increased production and stabilisation of α-D-glucosidase in the presence of D-glucose and butyrate (Van Hove *et al.,* 1996) and was explained by the decrease in pH of culture conditions resulting from the increase in lactic acid production. Here, stabilisation of rh-α-D-glucosidase was shown to be monosaccharide-specific with D-glucose having a greater effect on rh-α-D-glucosidase than D-galactose. D-Glucose also prevented aggregation and precipitation of rh-α-D-glucosidase during purification from CHO-K1 cell culture and improved the yield compared to a previously described method.

The increased production and purification yield for rh-α-D-glucosidase, with D-glucose, could substantially reduce the cost of rh-α-D-glucosidase preparation for ERT.

The ability of D-glucose to stabilise rh-α-D-glucosidase was specific as it was not observed for rh-4-sulphatase, and was only partially evident for another glycosidase rh-α-L-iduronidase. This was not unexpected as 4-sulphatase is structurally quite different to α-D-glucosidase. However, α-L-iduronidase and α-D-glucosidase share a common catalytic mechanism and structural element (Henrissat, 1991; Jenkins *et al.,* 1995; Durand *et al.,* 1997; Coutinho and Henrissat, 1999; Durand *et al.,* 2000). Kinetic experiments showed D-glucose was an uncompetitive inhibitor of rh-α-L-iduronidase, suggesting it did not bind directly to the active site as observed with α-D-glucosidase, but rather in a related site (e.g. substrate binding site). In addition to this, D-glucose has opposite stereochemistry (at carbon five on the sugar ring; Figure 5.14) than that required for optimal binding to α-L-iduronidase and may be another factor contributing to the difference in stabilisation observed for rh-α-L-iduronidase and rh-α-D-glucosidase in culture, as evident in the time course experiments.

Since D-glucose was shown to bind to the α-D-glucosidase active site, it was proposed that the difference in antibody reactivity seen in animals treated with rh-α-D-glucosidase purified from transgenic rabbits milk (high antibody titres and high affinity epitope reactivity; section 4.1, Figures 5.9a, 5.10) compared to those treated with rh-α-D-glucosidase purified from CHO-K1 culture medium (low antibody titres and low-affinity epitope reactivity; Figures 5.9b and 5.11) was possibly a result of the D-glucose used during purification of rh-α-D-glucosidase from the CHO-K1 cell culture. Investigations in

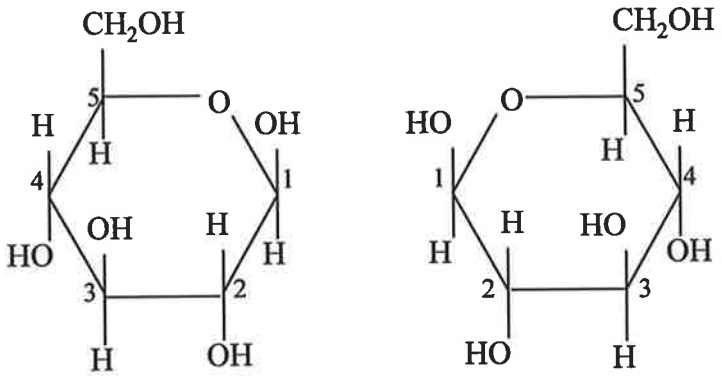

Figure 5.14: Stereochemistry of glucose

The D- and L-forms of a molecule (e.g. glucose shown above) are non-superimposable *mirror images* of each other and are stereoisomers called enantiomers. The D and L refers to the configuration of the highest numbered chiral carbon (i.e carbon 5). Enantiomers possess identical physical properties except when subjected to a chiral environment such as the human body. For example the stereospecificity of an enzyme will determine the degree of interaction exhibited with each enantiomer (i.e. a molecule with D-stereochemistry may not interact as strongly with an enzyme that has preference for molecules with L-stereochemistry).

an *in vitro* system showed a reduction in antibody reactivity to rh-α-D-glucosidase from CHO-K1 culture and from transgenic rabbit milk in the presence of 6 mg/mL and 50 mg/mL of D-glucose. Evaluation of rh-4-sulphatase and rh-α-L-iduronidase indicated high glucose concentrations (50 mg/ml) also mediated a reduction in antibody reactivity against these two enzymes, and this was possibly a viscosity effect. However, 6 mg/mL of D-glucose did not reduce antibody reactivity against rh-4-sulphatase and rh-α-L-iduronidase but did for rh-α-D-glucosidase. D-Glucose or its analogues may therefore be beneficial in minimising the antibody reactivity against α-D-glucosidase.

The ability of D-glucose to stabilise endogenous rh-α-D-glucosidase in CHO-K1 cells, suggested that a similar strategy could be used to enhance the level of α-D-glucosidase activity in GSD II cells. In fibroblasts from GSD II patients, only those with significant levels of α-D-glucosidase protein responded to D-glucose (6.0 g/L) treatment. D-Glucose treatment of GSD II skin fibroblasts from adult-onset patients resulted in an increase in residual α-D-glucosidase protein and activity providing proof of principal for the use of a D-glucose analogue in EET. EET has already shown promising results in preclinical trials of four other LSD: Gaucher disease/β-glucosidase (Sawkar *et al.,* 2002), MPS IVB/β-galactosidase (Matsuda *et al.,* 2003), Fabry disease/α-D-galactosidase A (Fan *et al.,* 1999) and Tay-Sachs disease and Sandhoff disease/β-hexosaminidase (Tropak *et al.,* 2004). Infusion of D-galactose in a 55 year old Fabry patient (deficient in α-D-galactosidase) increased residual activity of the enzyme 1.4-fold (from 7 to 10% of normal values) in lymphocytes and this was enough to significantly ameliorate the patient's condition (Frustaci *et al.,* 2001). Here, the treatment of adult-onset GSD II skin fibroblasts with D-glucose resulted in a 1.5-fold increase in residual α-D-glucosidase activity consistent with

the trend seen in the D-galactose infused Fabry patient. Analogues of D-glucose that are more potent inhibitors of α-D-glucosidase would be ideal candidates for EET in GSD II patients who have residual mutant enzyme activity.

This study demonstrated that D-glucose stabilised rh-α-D-glucosidase allowing increased expression and improved purification of the recombinant protein. D-Glucose enhanced the residual α-D-glucosidase protein/activity in adult-onset GSD II patient cells and suggested that the development of a D-glucose analogue may prove useful for EET. D-Glucose-treated rh-α-D-glucosidase had reduced antibody reactivity suggesting that it may also be useful as a modifier of the immune response to ERT in GSD II patients. In addition to this, it is possible that the reduced aggregate and precipitate formation of rh-α-D-glucosidase in the presence of D-glucose could also influence the antigenicity of this lysosomal protein.

CHAPTER 6:

CONCLUDING DISCUSSION

AIMS OF THIS STUDY

ERT by intravenous infusion has been shown to be effective for treating somatic tissue pathology, and is now in clinical practice for some LSD. However, a significant proportion of ERT-treated LSD patients have developed a humoral immune response to the replacement protein (Table 1.2). The level of this response is influenced partly by the inherent antigenicity of the infused protein but can also depend on the individual patient. The potential problems associated with a humoral immune response to enzyme infusion include hypersensitivity reactions, altered enzyme traffic, altered targeting and neutralisation of enzyme activity. Antibody development against the replacement protein is therefore an important issue for LSD patients undergoing ERT.

The high frequency of antibody development in enzyme-treated LSD patients raises several critical questions: what promotes antibody development to the replacement protein in LSD patients? Are there any common factors between replacement proteins that contribute to humoral immune response? How long is the antibody response maintained? This thesis presented a hypothesis that specific epitopes, which may be conserved in related enzymes, are involved in the initiation and maintenance of antibody reactivity to ERT in LSD patients. The aim of this thesis was to define the progression and molecular basis of antibody responses against two distantly related lysosomal enzymes, α-L-iduronidase and α-D-glucosidase (deficient in MPS I and GSD II respectively). Characterising the antibody reactivity to these two lysosomal enzymes could aid in developing strategies to avert the potential adverse effects of antibody production.

ANTIBODY DEVELOPMENT TO ERT

Antibody development to ERT has been reported in animal models of LSD. After 12-months of treatment with rh-4-sulphatase in MPS VI cats, high-affinity antibodies to 4-sulphatase were detected in 30% of the treated animals (Brooks *et al.,* 1997; Turner *et al.,* 1999). In 100% of GSD II mice (a knockout model) treated with rh-α-D-glucosidase (Raben *et al.,* 2003) and 100% of MPS I dogs (null mutation), treated with rh-α-L-iduronidase, (Shull *et al.,* 1994; Kakkis *et al.,* 1996) developed antibody responses to the replacement protein. The MPS VI cats have two mutations (D520N and L476P) that result in a range of residual 4S activity from 0.5% to 4.6% of normal levels (Yogalingam *et al.,* 1998). The low levels of residual 4-sulphatase in the MPS VI cat model may have been sufficient to prime the immune system and consequently result in a lower antibody response to ERT when compared to the knockout and null models of GSD II and MPS I, that have no residual protein and would be considered immunologically naïve. In contrast to MPS VI cats treated by ERT at a later age, cats treated from birth developed negligible antibody titres to the administered rh-4-sulphatase protein (Auclair *et al.,* 2003) suggesting the development of antibodies could be prevented by early treatment when the immune system is more amendable to tolerance induction. It was reported in this thesis that 100% of MPS I mice (knockout model) that do not have any mutant α-L-iduronidase, developed high antibody titres to rh-α-L-iduronidase ERT (Chapter 3). However, antibody titres in MPS I immune-deficient mice were within the normal control range. The animal model studies indicated a high potential for antibody development in response to ERT, in particularly both in the absence of residual mutant enzyme and when therapy is initiated at a later age.

Antibody responses have also been reported in ERT-treated LSD patients. In a phase I/II clinical trial in MPS I patients receiving rh-α-L-iduronidase ERT, 50% of patients developed high levels of antibody to the infused protein (Chapter 3; Kakavanos *et al.,* 2003); in a further clinical trial, 91% of MPS I patients developed antibodies to rh-α-L-iduronidase (EC 3.2.1.76, Aldurazyme®, 2003). In Gaucher patients receiving rh-β-glucocerebrosidase 13% of patients demonstrated an immune response to the infused protein (Rosenberg *et al.,* 1999; Cerezyme®, 2002). Evidence of an antibody response was detected in 83% of Pompe patients treated with rh-α-D-glucosidase (EC 3.2.1.20) purified from CHO culture (http://www.worldpompe.org/myozyme260405.html). To date all patients treated with rh-α-D-glucosidase purified from transgenic rabbit milk developed higher levels of antibodies against the replacement enzyme compared to placebo-treated controls (Van den Hout *et al.,* 2004; Winkel *et al.,* 2004; Klinge *et al.,* 2005). In a phase I/II open label extension study in Fabry patients receiving rh-α-D-galactosidase A (EC 3.2.1.22, Fabrazyme®), 88% of patients developed antibody titres to the infused protein (Eng *et al.,* 2001; Fabrazyme®, 2003); in a subsequent clinical trial 55% of patients developed antibody titres to rh-α-D-galactosidase A (Replagal®, 2001). One hundred percent of MPS VI patients receiving rh-*N*-acetylgalactosamine 4-sulphatase (EC 3.1.6.12, Aryplase®, 2003) showed evidence of an antibody response to this replacement protein. The potential for immune response to ERT in LSD patients has proven high and presents as the only major complication from enzyme administration. This initiated further investigation on the characterisation of the humoral immune response against ERT in LSD patients.

DURATION OF THE HUMORAL IMMUNE RESPONSE TO ERT IN LSD PATIENTS

In a phase I/II clinical trial of ERT the progression of antibody response to rh-α-L-iduronidase was characterised in MPS I patients. Before initiating ERT, background antibody titres were detected in all MPS I patients (Chapter 3; Kakavanos *et al.,* 2003). Background antibody titres to other lysosomal proteins have also been reported in other LSD (Brooks *et al.,* 1997; Daly *et al.,* 2000; Kakkis *et al.,* 2001; Linthorst *et al.,* 2004). In immune responsive MPS I patients, antibody titres increased to higher than background levels after ERT was initiated, with maximum levels observed between 12- to 26-weeks of therapy (Chapter 3, Kakavanos *et al.,* 2003). Patients who generated an immune response showed a decline in titres to rh-α-L-iduronidase after six-months of ERT, and developed immune tolerance by one-year of therapy. This trend towards immune tolerance has also been observed in other LSD patients receiving ERT: for example, all patients treated with rh-α- D-glucosidase purified from transgenic rabbit milk developed high antibody titres against the replacement enzyme during the first 20- to 48-weeks of therapy compared to control levels, but these titres declined with subsequent infusions (Van den Hout *et al.,* 2004; Winkel *et al.,* 2004). Similarly, reduced antibody titres to rh-α-D-galactosidase have been observed in immune-responsive Fabry patients after a year of therapy (Eng *et al.*, 2001; Schiffmann *et al.*, 2001). These observations have positive implications for patients receiving long-term ERT because the low antibody reactivity resulting after immune tolerance would have minimum impact on the efficacy of therapy and the health of the patient. This was also consistent with the reduced number of hypersensitivity reactions experienced by the immune-responsive MPS I patients after long-term ERT (Kakkis *et al.,* 2001; Kakavanos *et al.,* 2003; Wraith, 2005). Thus, although some LSD patients

undergoing ERT develop a high level of antibody reactivity to the replacement protein, this reactivity has not tended to persist beyond 12-months of ERT treatment.

The majority of the MPS I patients involved in the phase I/II clinical trial had a slowly progressing clinical phenotype (Hurler-Scheie and Scheie phenotypes), it would therefore be of particular interest to investigate the humoral immune response to ERT in MPS I patients with null mutations (no protein, Hurler phenotype eg W402X, Q70X mutations) that would be expected to mount a more aggressive immune response to ERT. Studies of ERT in animal models with null genotypes confirm a higher level of antibody development (Shull *et al.,* 1994; Chapter 3) and this has also been observed in infantile-onset GSD II patients (Amalfitano *et al.,* 2001; Kishnani and Howell, 2004; Van den Hout *et al.,* 2004). Furthermore, it is not yet clear whether all enzyme-treated LSD patients that mount an immune response will develop immune tolerance, particularly those with null mutations. A recent study of ERT in Fabry patients with a null genotype showed 69% developed an immune response but only 58% developed immune tolerance after prolonged ERT (Linthorst *et al.,* 2004). Therefore there is still a need to further characterise the humoral immune response to ERT in LSD patients.

EPITOPE REACTIVITY OF ANTIBODIES PRODUCED IN RESPONSE TO ERT

I hypothesised that specific epitopes on replacement proteins were involved in the development of high affinity antibody reactivity during enzyme treatment in LSD. In MPS I patients receiving ERT, four linear sequence peptide regions on α-L-iduronidase showed common reactivity in the immune-responsive patients; one of these, peptide 19 on α-L-iduronidase, showed the most sustained reactivity and was the last to tolerise (Chapter 3;

Kakavanos *et al.,* 2003). The appearance of high affinity epitopes across the α-L-iduronidase protein coincided with the peak in antibody titre levels detected in the immune-responsive MPS I patients. After eight-weeks of rh-α-D-glucosidase treatment, rodents developed high levels of antibody and multiple high affinity epitopes were observed across the protein (Chapters 4 and 5); several high affinity epitopes were identified in most of the ERT-treated animals. Following 4-sulphatase ERT, an increase in the number of linear sequence epitopes was observed in MPS VI cats, with some suggestion of specific epitope involvement (Turner *et al.,* 1999). In a rat model of MPS I, antibody reacting to a single linear sequence epitope altered the tissue distribution of infused rh-α-L-iduronidase enzyme (Glaros *et al.,* 2002). In a number of LSD evidence suggests that specific linear sequence epitopes play a significant role in the development and maintenance of an immune response to ERT.

COMMON ANTIGENICITY IN LYSOSOMAL GLYCOSIDASES

I hypothesised that conserved epitopes or common structures could be involved in the development of an immune response to ERT for different glycosidases. Antibody reactivity to rh-α-L-iduronidase showed that high affinity epitopes were located in the first half (N-terminus) of the protein sequence. These epitopes mapped to the predicted location of the outer α-helix elements of the α-L-iduronidase $(\beta/\alpha)_8$ barrel (Henrissat *et al.,* 1995; Durand *et al.,* 2000; Brooks *et al.,* 2001; Kakavanos *et al.,* 2003). Significant cross-reactivity was observed for antibodies generated to α-L-iduronidase, as well as α-D-glucosidase, which also has a $(\beta/\alpha)_8$ barrel catalytic element (Chapter 4, Kakavanos *et al.,* 2006). This study demonstrated that conserved micro-structural features and regions of short sequence identity could contribute to common glycosidase antigenicity. This may explain the background antibody titres observed in LSD patients prior to the onset of ERT, particularly

in those patients with null mutations (Brooks *et al.,* 1997; Daly *et al.,* 2000; Kakkis *et al.,* 2001; Kakavanos *et al.,* 2003; Linthorst *et al.,* 2004). The presence of antibodies to lysosomal enzymes normally in circulation suggested that they may have a biological role. It was speculated that this may include a mechanism for removing lysosomal proteins released into circulation where they might act inappropriately on their natural substrates in the extracellular matrix. I hypothesised that intravenous ERT may disturb the balance of endogenous immune reactivity (produced in response to a cross-reacting antigen), contributing to antibody reactivity in some LSD patients. This may not represent a new immune response and, over time, tolerance to these reactive antigens could be restored, as observed in MPS I patients receiving replacement enzyme (Chapter 3, Kakavanos *et al,* 2003). Therefore, common glycosidase antigenicity may also account for why immune response to ERT has proven to be less of an issue than initially postulated.

AVERTING THE ADVERSE EFFECTS OF IMMUNE RESPONSE TO ERT

In human clinical trials of ERT there has been a relatively low incidence of neutralising antibodies. Neutralising antibody, causing a regression in clinical improvement, has been reported in <0.5% of Gaucher patients (Brady *et al.,* 1997). In two Gaucher patients who developed an immune response to the infused enzyme it was reported that the antibodies inactivated the normal wild-type protein but not the patients' N370S mutant form of the protein (Pastores *et al.,* 1993). In a recent study of antibody reactivity in enzyme-treated Fabry patients, 69% developed neutralising sera antibodies to the α-D-galactosidase replacement protein, which coincided with continued urinary excretion of globotriaosylceramide substrate (Linthorst *et al.,* 2004). Evidence of the development of neutralising antibody to a replacement protein has also been reported for infantile-onset

GSD II. Two of three infantile-onset GSD II patients treated with rh-α-D-glucosidase purified from CHO cells in a phase I/II clinical trial, showed a decline in muscle function that coincided with the appearance of anti-α-D-glucosidase antibodies (Amalfitano *et al.,* 2001). In contrast, a third patient who continued to improve did not develop anti-α-D-glucosidase antibodies. Although only a minority of ERT-treated LSD patients have developed neutralising antibodies, even subtle decreases in the efficacy of treatment could still be mediated by antibody production and potentially alter the dose required for effective therapy.

Animal studies have further demonstrated some of the potential effects of antibody production on ERT. A 4-sulphatase distribution study performed in rats showed that high titre antibodies resulted in increased amounts of enzyme in the lungs at the tissue level and in lower density (endosomes) organelles at the subcellular level (Brooks *et al.,* 1998); moreover, rapid removal and degradation of the infused rh-4-sulphatase was observed in high- compared to low-titre rats. In addition, plasma from ERT-treated MPS VI cats with high antibody titres inhibited 47% of rh-4-sulphatase *in vitro* (Auclair *et al.,* 2003). Significant clearance of lysosomal storage was reported within many cell types in all treated MPS VI cats but this was slightly less pronounced in some tissues for MPS VI cats with high antibody titres (Auclair *et al.,* 2003).

In an immunized rat model of α-L-iduronidase, a high level of antibody was required to induce changes in targeting and distribution, and resulted in increased levels of this enzyme in lung, kidney and plasma, when compared to controls (Turner *et al.,* 2000). Altered subcellular trafficking to lysosomes was evident in high titre rats but in this case appeared to enhance the activity of rh-α-L-iduronidase. In MPS I dogs, rh-α-L-iduronidase

ERT decreased lysosomal storage and improved clinical outcome regardless of the presence of antibodies to the α-L-iduronidase replacement protein (Shull *et al.,* 1994; Kakkis *et al.,* 1996). However, the development of antibodies to rh-α-L-iduronidase was associated with a complement-mediated anaphylactoid reaction if enzyme infusion was too rapid (Shull *et al.,* 1994) and significant proteinuria (Kakkis *et al.,* 1996). These animal studies showed that high levels of antibody can have some effect on enzyme targeting, intracellular traffic and turnover. However, it should be noted that there have been no negative effects on ERT efficacy reported for MPS VI and MPS I patients treated by ERT thus far although these are adhoc observations and are no in comparison to a control. Nevertheless, it would be beneficial to be able to predict immune reactivity and to prevent the development of antibodies against replacement proteins.

Prediction of immune reactivity resulting from the delivery of enzyme in a LSD patient is important when considering the management (slowing the infusion rate, administering antihistamines etc.) and safety of patients. In enzyme-treated MPS I patients, a weak inverse correlation was noted between residual protein concentration detected in skin fibroblast cells (Yogalingam *et al,* 2004) and the degree of immune response (Chapter 3; Kakavanos *et al.,* 2003). Patients with null mutations (no residual mutant protein) would therefore, be expected to mount a more vigorous immune response to ERT that those with other genotypes. Therefore, the level of residual mutant protein in untreated LSD patients could act as a partial predictor of immune response to ERT and allow early detection and subsequent action to minimise or prevent antibody reactivity.

Currently, a protocol employed to address the adverse effects of antibody production in patients is the induction of immune tolerance by antigen administration. This technique can

be aggressive, costly and invasive (Brady *et al.,* 1997; Ponce *et al.,* 1997; Rosenburg *et al.,* 1999). For example, a strategy for managing a Gaucher patient who had developed neutralising antibodies has been reported and includes treatment with plasma exchange to reduce the concentration of circulating antibody, cyclophosphamide administration to kill reactive B cells, intravenous immunoglobulin G infusion to inhibit immune complex formation and control immune reactivity, and high dose enzyme administration to induce immune tolerance (Brady *et al.,* 1997). Alternative management or prevention strategies are being developed. Recently, a tolerance regimen using a combination of immunosuppressive drugs was described for a canine model of MPS I (Kakkis *et al.,* 2004). Other strategies to avert the adverse effects of antibody production may also be engineered by defining the reactive epitopes on the replacement protein. This may involve the induction of tolerance to specific epitopes (such as those identified in this thesis) before therapy is initiated, or infusion of short linear sequence peptides prior to enzyme infusion to deplete the circulation of anti-replacement enzyme antibodies. However, further investigation is required to validate these strategies for the management of immune responses to ERT.

An alternative strategy for the management of the immune response to ERT in LSD patients is the prevention of antibody interaction to key antigenic sites on replacement proteins. The catalytic site of α-D-glucosidase was shown to be antigenic (Chapters 4 and 5; Kakavanos *et al.,* 2006) and antibodies binding to the catalytic site would be expected to neutralise α-D-glucosidase activity. Infantile-onset GSD II patients have a rapidly progressive clinical phenotype and die by one-year of age if untreated (Hirschhorn and Reuser, 2001). Consequently, neutralisation of α-D-glucosidase activity during ERT would be extremely detrimental to this patient group. The monosaccharide D-glucose was shown

to bind the α-D-glucosidase active site and reduced antibody reactivity against α-D-glucosidase i*n vitro* (Chapter 5). This simple and inexpensive strategy may be more attractive for reducing the immune response to ERT in GSD II patients than some of the tolerance strategies currently in place.

CONCLUDING STATEMENT

It is clear that some LSD patients will develop antibodies in response to ERT, which could potentially compromise the safety of the patient and the efficacy of therapy. Therefore, characterising these immune responses becomes crucial for the management of patients receiving long term ERT. This thesis reports the immune reactivity of two lysosomal glycosidases; α-D-glucosidase and α-L-iduronidase. I have shown that high titre and high affinity antibody responses are generated in some patients, usually by 26-weeks of ERT; in some cases hypersensitivity reactions may also occur during this time. However, the majority of immune-responsive patients have eventually developed immune-tolerance by one- year of therapy. Specific linear sequence epitopes have been implicated in the development and maintenance of this humoral immune response to ERT in LSD patients. I have also reported evidence to suggest that there are common epitopes among structurally related lysosomal enzymes that may contribute to antibody reactivity during ERT in some patients. This work will assist the long-term aim of delivering a more effective and safe ERT for patients who develop an immune response. This includes the ability to predict and monitor immune reactivity in LSD patients and the identification of potential strategies to avert the adverse effects of antibody production to ERT.

FURTHER RESEARCH

Epitope mapping has determined the antigenic regions on two glycosidase enzymes, α-L-iduronidase and α-D-glucosidase. Specific linear sequence epitopes appeared to be important for the development and maintenance of a humoral immune response against these two replacement proteins. Therefore, the role of individual epitopes, particularly those that are cross-reactive between glycosidases, should be further characterised. One study with α-L-iduronidase has already shown that a monoclonal antibody reacting to a single epitope was capable of altering organ distribution of the infused enzyme (Glaros *et al.,* 2002). Furthermore, there is a need to have controlled experiments to test the effect of antibodies on the efficacy of ERT.

Further research to determine the presence or absence of cross-reactive antigenic motifs in other structurally related lysosomal enzymes would be beneficial. Reactivity of antibodies from ERT-treated MPS VI cats to linear sequence epitopes has been investigated and mapped onto structural models of 4-sulphatase (Turner *et al.,* 1999). The lysosomal sulphatases have sequence and structural homology (Bond *et al.,* 1997) and therefore would be ideal candidates to investigate common antibody reactivity. Two sulphatases of interest would be iduronate-2-sulphatase and arysulphatase A, which are respectively deficient in the LSD MPS II and metachromatic leukodystrophy.

Strategies to reduce antibody reactivity against replacement enzymes are important for immune-responsive patients. The ability of specific linear sequence peptides to avert the adverse effects of antibody production should be investigated. Co-infusion experiments of synthesised peptides with enzyme in a high-titre animal model would determine the ability of the administered peptides to avert altered subcellular distribution caused by high affinity

antibodies. D-Glucose has been shown to inhibit antibody reactivity against the catalytic site of α-D-glucosidase *in vitro*, but its effect *in vivo* needs to be characterised to evaluate its usefulness in reducing antibody reactivity to ERT in GSD II patients. Other target molecules that could prevent antibody-antigen interaction at key sites of antigenicity on other lysosomal enzymes should be investigated.

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Revecca Kakavanos: PhD Thesis Addendum
Immune Response to Enzyme Replacement Therapy in MPS I and GSD II Patients
Assessor #1:
Page 25: Figure 1.6 Labels should read α-L-iduronidase and N-Acetyl galactosamine
Page 36: ERT is also in clinical practice for Pompe disease.
Page 36; line13: mannose-6-phosphate receptor
Page 37; line 9: pre-clinical
Page 58; line 2: saline
Page 58; line 17: Hydrochloric
Assessor #2:
Page 6; last line: replace sentence with: "For example, of the total number of patients with Gaucher, Tay Sachs, Niemann-Pick type A diseases and mucolipidosis type IV, about 50% are of Ashkenazic Jewish ancestry."
Page 31; line 2: insert reference: Fuller et al., 2005 [Fuller, M., Brooks, D.A., Evangelista, M., Hein, L.K., Hopwood, J.J., Meikle, P.J. (2005): Prediction of neuropathology in mucopolysaccharidosis I patients. *Mol. Genet. Metab.* **84:** 18-24.].
Page 43; line 6: ….involved in inflammation,…..
Page 55; 6th line from bottom: delete ")."
Page 58; line 2: saline
Page 66; line 17: insert dose/rat in brackets: "; 25μg/kg"
Page 80; Figure 3.1 legend: insert (1, 3): ….MPS I patients 1, 3-5 and 10…….
Page 81; line 16: insert new sentence: "It was interesting to note that the pre-treatment levels of epitope reactivity for patients 6-9 were slightly higher compared to the other patients (Figures 3.2-3.6) and this may therefore be an indicator of the potential to develop an immune response."
Page 87; Figure legend: insert "See Figure 3.8 for the sequence location of the common epitope reactivities."
Page 91; Figure legend: insert "See Figure 3.7 for further details of epitope reactivity."
Page 107; Figure legend: fortnightly
Page 124; line 6: insert new sentence: "It could also reflect the very large tissue mass of the ERT target organ, muscle, in GSD II."
Page133; line 7: replace "evident" with "obvious" and insert new sentence: "However, from these data it would be premature to conclude that there was no stability effect for these sugars on rh-4-sulphatase".
Page 133; line 14: …in a cell-free assay system….
Page 154; line 3: change "observed" to "obvious".
Page 157; line 1: insert new sentence: "However, it would not be practical to infuse Pompe patients with high levels of D-glucose, due to the potential adverse effect on glucose metabolism."
Page 157; line 2: change "would' to "could".
Page 167; line 9: change "…and are not in comparison to a control." to "and were not compared to a control."
Page 167; line 9: insert new sentence: "Moreover, most studies to date have had very limited outcome measures and any reductions in efficacy of ERT would be difficult, if not impossible to detect".