Origin and features of mammalian chitinases
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GENERAL INTRODUCTION

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OUTLINE OF THE THESIS
Many organisms possess an exoskeleton, a macromolecular armor that provides protection from harsh environmental conditions and possible predators. Most of the animals with an exoskeleton are arthropods, including crabs, lobsters, spiders and insects. In arthropods the main constituent of the exoskeleton is chitin, a linear polymer of β-1,4-linked N-acetylglucosamine (GlcNAc) moieties. Natural chitin is an insoluble polymer of considerable mechanical and chemical strength, mainly due to hydrogen bonding between the >NH and the >C=O groups of the N-acetyl groups of aligning chitin polymers, that can occur in parallel (α), anti-parallel (β) or mixture (γ) of aligned chains. Due to the abundance of arthropods in nature, chitin is the second most abundant biopolymer, after cellulose. Besides arthropods, other organisms also synthesize chitin for structural purposes. Fungi, for instance, employ chitin as part of their cell wall, rendering them more resistant to the outside environment (Figure 1A). All chitin-containing organisms need to be able to break down their chitin to allow for remodelling. In the case of fungi for instance, chitin has to be locally removed in the cell wall for growth and division. The degradation of chitin is mediated by chitinases (E.C. 3.2.1.14), in concert with other chitinolytic hydrolases. Chitinases cleave within the chitin chains or remove oligosaccharides from the non-reducing end, and break down the polymer in combination with β-hexosaminidases (E.C. 3.2.1.52). Besides remodelling, chitinases are also made for other purposes. Plants, for example, although lacking chitin, produce chitinases. Evolution has equipped them with chitinases as protection against chitin-containing pathogens. Furthermore, several bacterial species produce chitinases in order to eventually release GlcNAc monomers that subsequently can be used for nutritional purposes.

Since chitin is not synthesized by mammals, chitinases were for a long time believed to be absent in these organisms. Serendipitously, the existence of true chitinases in mammals was firstly discovered by researchers in the Academic Medical Center as a result of their fundamental biochemical investigations on plasma specimens from patients suffering from Gaucher disease, a lysosomal storage disorder. Gaucher disease is a rare, recessively inherited disorder. The first detailed case report was published by Philippe E. Gaucher, over a century ago (Gaucher, 1882) (Figure 1B, inset). The incidence of the disorder has been estimated to be 1 in 50,000 to 200,000 births. Gaucher disease is caused by a deficient capacity to degrade the glycosphingolipid glucosylceramide. This lipid consists of a glucose moiety beta-glycosidically linked to a ceramide. Degradation of this lipid normally occurs predominantly inside lysosomes (Figure 1, background) by the sequential action of the acid β-glucosidase (glucocerebrosidase) and acid ceramidase. The activity of the former enzyme (GBA1; E.C. 3.2.1.45) is impaired in patients suffering from Gaucher disease (Brady et al., 1965; Patrick, 1965). Although glucocerebrosidase activity is
comparably reduced in all cell types of Gaucher patients, the lysosomal storage of

glucosylceramide is restricted to cells of the monocyte/macrophage lineage, at

least in the type 1 variant (Beutler and Grabowski, 2001). The predominant lipid
accumulation in macrophages can be ascribed to the role of these cells in
degradation of senescent red and white blood cells that are rich in

glycosphingolipids (Parkin and Brunning, 1982; Naito et al., 1988). The storage
cells show a characteristic morphology with an eccentric nucleus and a “wrinkled
tissue paper” like appearance due to the massive presence of lipid in tubular
deposits. These storage cells are referred to as Gaucher cells and are present in
various locations, predominantly the spleen, liver, bone marrow and parenchyma
of lymph nodes (Figure 1B). As a result, Gaucher patients develop gross
hepatosplenomegaly (Figure 1C). In the bone marrow, the massive accumulation
of storage cells causes displacement of the normal haematopoietic cells. In other
tissues, infiltration of Gaucher cells may lead to infarction, necrosis and fibrous
scarring. The sheer presence of storage cells does not fully explain the entire
pathology of Gaucher disease. Gaucher cells are not inert storage containers, but
metabolically active cells that are produce and secrete proteins that could drive
pathophysiological processes. It is now generally believed that the complex
mixture of factors, like cytokines, chemokines and hydrolases, originating from
storage cells themselves or from surrounding macrophages contributes to the
characteristic pathophysiology of Gaucher disease (Boven et al., 2004).

Immediately after his discovery of lysosomes, Christian de Duve proposed that
treatment of lysosomal storage disorders by supplementation with the missing
enzyme might be feasible (de Duve, 2005). Inspired by this concept, Brady and
co-workers at the National Institutes of Health elegantly exploited the presence of
the so-called mannose receptor on the cell surface of macrophages to target
therapeutic enzyme to lysosomes of lipid-laden macrophages in Gaucher patients.

For this purpose, the oligosaccharide chains of glucocerebrosidase isolated from
placenta were modified by enzymatically exposing the covered mannose residues
(Furbish et al., 1981). The concept of a mannose-terminated glucocerebrosidase
resulted in the development of the registered therapeutic enzyme alglucerase
(Ceredase, Genzyme Corporation, MA). Intravenous administration of alglucerase
was found to result in major clinical improvements (Barton et al., 1990; Barton et
al., 1991). Ceredase was replaced in the mid-nineties by recombinant produced
glucocerebrosidase from CHO cells (Cerezyme), with similar therapeutic results
(Grabowski et al., 1995).

An alternative approach for therapeutic intervention of type 1 Gaucher and other
glycosphingolipid storage disorders is substrate reduction therapy (SRT; also
termed substrate deprivation therapy; reviewed in Radin, 1996; Aerts et al., 2006).
The approach aims to reduce the rate of glycosphingolipid biosynthesis to levels
which match the impaired catabolism. Two classes of inhibitors of
glycosphingolipid biosynthesis have presently been described, both of which
inhibit the ceramide-specific glucosyltransferase, (also termed glucosylceramide synthase; GlcT-1; UDP-glucose: N-acylsphingosine D-glucosyl-transferase, EC 2.4.1.80). The enzyme catalyses the first step in the biosynthesis of glucosphinolipids: the transfer of glucose to ceramide. The first class of inhibitors is formed by analogues of ceramide. The prototype inhibitor is PDMP (D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol). More specific and potent analogues have been subsequently developed (Shayman et al., 2004; McEachern et al., 2007). The second class of inhibitors of glucosylceramide synthase is formed by N-alkylated iminosugars. Such type of compounds were already in common use as inhibitors of N-glycan processing enzymes and the potential application of N-butyldeoxynojirimycin as HIV inhibitor had been studied in AIDS patients. Platt and Butters at the Glycobiology Institute in Oxford were the first to recognize the ability of N-butyldeoxynojirimycin (NB-DNJ) to inhibit glycosylceramide synthesis at low micromolar concentrations (Platt et al., 1994). Overkleeft and coworkers in their search for inhibitors of glucosidases have serendipitously developed a more potent inhibitor of glucosylceramide synthase. Adamantane-pentyl-deoxynojirimycin (AMP-DNM) was found to inhibit glycosphinolipid biosynthesis at low nanomolar concentrations (Overkleeft et al., 1998) and to prevent globotriaosylceramide accumulation in a Fabry knock out mouse model without overt side effects (Aerts et al., 2003). Oral administration of N-butyldeoxynojirimycin to adult type 1 Gaucher patients resulted in improvements in hepatosplenomegaly and hematological abnormalities as well as corrections in plasma levels of glucosylceramide (Cox et al., 2000). NB-DNJ (Zavesca, Actelion) is now registered in Europe and the U.S.A. for treatment of mild to moderately affected type 1 Gaucher patients that are unsuitable to receive enzyme replacement therapy (Cox et al., 2003). Sustained effects of prolonged substrate reduction therapy have recently been documented (Pastores et al., 2005; Elstein et al., 2007).

The availability of costly therapeutic interventions for Gaucher disease soon prompted a search for biomarkers of Gaucher cells that would allow accurate monitoring of the progress of the disease and efficacy of therapy. The ideal biomarker is detectable in plasma and directly reflects the presence of storage cells. Although abnormalities in plasma levels of tartrate resistant acid phosphatase (TRAP), angiotensin-converting enzyme (ACE), hexosaminidase and lysozyme have all been reported, none of these enzymes appear to meet this criterion (reviewed in Aerts and Hollak, 1997). Overlap between levels of these enzymes in patients versus controls further restricts their use as biomarkers in Gaucher disease. In an attempt to identify novel secondary biochemical abnormalities, a thorough screening of plasma enzyme activities in plasma of symptomatic individuals versus a variety of substrates was conducted. This led to the discovery in plasma of Gaucher patients of a thousand-fold increased capacity to hydrolyse the fluorogenic substrate 4-methylumbelliferyl–chitotrioside (Hollak et
The responsible enzyme was named chitotriosidase. Further studies revealed that plasma chitotriosidase originated from the lipid-laden macrophages of Gaucher patients. As a result of this, chitotriosidase activity levels do not reflect one particular clinical symptom, but rather reflect the total body burden on Gaucher cells. Although chitotriosidase activity can be rapidly and sensitively measured using 4-methylumbelliferyl-chitotrioside as substrate, the occurrence of substrate inhibition due to the ability of the enzyme to transglycosylate complicates the enzyme assay. The use of a slightly modified substrate provides a much more convenient method for measuring activity of chitotriosidase (Aguilera et al., 2003; Schoonhoven et al., 2007).

Plasma chitotriosidase activities are greatly increased in symptomatic Gaucher patients, but not in asymptomatic glucocerebrosidase-deficient individuals. Chitotriosidase values drop sharply upon ERT, coinciding with clinical improvements (Hollak et al., 1994). To assess the utility of chitotriosidase activity measurements as a biomarker for treatment efficacy, its relationship with clinical parameters has been studied (Hollak et al., 2001). On the basis of this investigation, it has been proposed that in patients in whom initiation of treatment is questionable, based solely on clinical parameters, a chitotriosidase activity above 15,000 nmol/ml*hour may serve as an indicator of a high Gaucher cell burden and an additional indication for the initiation of treatment. A reduction of less than 15% after one year of treatment should be a reason to consider a dose increase. Furthermore, a sustained increase in chitotriosidase at any point during treatment should alert the physician to the possibility of clinical deterioration and the need for dose adjustment, and hence are of great potential in both diagnosis and monitoring of the disease. The regular monitoring of plasma chitotriosidase levels in Gaucher patients is presently used world-wide to assist in clinical management of these patients (Deegan et al., 2005; Vellodi et al., 2005; Cabrera-Salazar et al., 2004). A pitfall regarding the use of chitotriosidase as Gaucher cell biomarker results from the complete absence of the chitotriosidase activity in about 6% of all individuals, including Gaucher patients. This results from homozygosity for a null allele of the chitotriosidase gene (Boot et al., 1998). Plasma chitotriosidase levels in heterozygotes for this mutation (about 35% of all individuals) underestimate the actual presence of Gaucher cells in patients. Although plasma chitotriosidase activity is now the mostly used biomarker in GD, there is still need for other biomarkers foremost because of the high incidence of deficiency. Recently a marked elevation in plasma of GD patients has also been described for the chemokine CCL18 (Boo et al., 2004; Moran et al., 2000; Deegan et al., 2005). Both chitotriosidase and CCL18 are secreted by Gaucher cells and both the plasma levels of both markers change comparably during therapy. Monitoring of plasma CCL18 can therefore be a useful alternative to monitor response to therapy in Gaucher patients deficient in chitotriosidase (Deegan et al., 2005).

The serendipitous discovery of chitotriosidase in Gaucher patients has been the
starting point of a long series of fundamental studies on this chitinase and related proteins that are present in man and other mammals. This thesis describes the latest studies in this respect. In chapter 2, a detailed overview is presented of the initial work of AMC researchers on characterization of chitotriosidase, the related acidic mammalian chitinase (AMCase) and chi-lectins. Briefly, chitotriosidase was firstly purified from spleen of a Gaucher patient (Renkema et al., 1995). Two major isoforms with molecular masses of 50 and 39 kDa were isolated and both were shown to be functional chitinases, exhibiting activity towards colloidal chitin as well as artificial fluorogenic substrates. Their activity could be inhibited by allosamidin and demethyl allosamidin, in a manner similar to bacterial chitinases (Renkema et al., 1995). Using degenerate primers based on conserved regions in chitinases from several species, the corresponding cDNA was cloned from a macrophage cDNA library (Boot et al., 1995). Sequence alignments showed that chitotriosidase is remarkably homologous to chitinases of various species, belonging to family 18 of glucosyl hydrolases; in particular the catalytic region consensus sequence (D-x-x-D-x-D-x-E) is completely conserved. Alignment of chitotriosidase with other chitinases also showed that the enzyme consists of a 39-kDa catalytic domain connected with a C-terminal chitin binding domain through a short linker region, again in a manner similar to other chitinases. It was found that chitotriosidase is synthesized as a 50-kDa protein that is either secreted into the medium or, alternatively, processed into the 39-kDa enzyme in the lysosome where it

Figure 1. Overview of features relating to Gaucher disease and chitinases (see text for details).
accumulates. To a quantitatively minor extent, a 39-kDa isoform containing only one extra C-terminal residue can also be synthesized as a result from alternative splicing (Renkema et al., 1997; Boot et al., 1995). Next, the locus of the chitotriosidase gene was assigned to 1q31-32 by fluorescent in situ hybridization using the genomic clone as a probe (Boot et al., 1998). The commonly encountered recessively inherited deficiency in chitotriosidase could be attributed to a 24-base pair duplication causing aberrant splicing (Boot et al., 1998). The high incidence of the chitotriosidase deficiency in man prompted questions concerning redundancy of chitotriosidase. Further investigations led to the discovery of a second mammalian chitinase named Acidic Mammalian Chitinase (AMCase) (Boot et al., 2001). Similar to chitotriosidase, AMCase shows chitinolytic activity towards chitin, releasing mainly soluble chitobiose fragments and is sensitive to inhibition by allosamidin. Like chitotriosidase, AMCase is synthesized as a 50 kDa protein that contains a 39 kDa catalytic domain, separated from a C-terminal chitin binding domain by a hinge region. Although the sequence similarity between the human chitinases is high, AMCase exhibits a distinct pH activity profile, being most active at acidic pH (Boot et al., 2001).

The crystal structures of the native 39 kDa human chitotriosidase and complexes with a chitooligosaccharide and allosamidin have been resolved (Fusetti et al., 2002; Rao et al., 2003). The core domain has a (β/α)8 (TIM) barrel as observed in the other family 18 chitinase structures for hevamine, chitinases A (ChiA) and B (ChiB) from S. marcescens, and CTS1 from Coccidioides immitis, although helix α1 is missing (Fusetti et al., 2002; Figure 1D). An additional ω/β domain, composed of six antiparallel β-strands and one α-helix, is inserted in the loop between strand β7 and helix α7, which gives the active site a groove-like structure. Like all other family 18 chitinases, chitotriosidase has the D-x-x-D-x-D-x-E motif at the end of strand β4 with Glu140 being the catalytic acid. Two disulfide bridges were observed between residues 26-51 and 307-370. The crystal structures reveal an elongated active site cleft, compatible with the binding of long chitin polymers. Given the relatively open active site architecture, chitotriosidase appears to function as an endochitinase rather than an exochitinase. The complex with GlcNAc2, followed by modeling of a longer chitooligosaccharide revealed that the active site would be able to accommodate longer chitin polymers, in agreement with its ability to degrade various forms of polymeric chitin.

Besides the active chitinases, highly homologous mammalian proteins lacking enzymatic activity due to substitution of active site catalytic residues have been identified. Despite their lack of enzymatic activity, these proteins have retained active site carbohydrate binding, and hence have been named chi-lectins (Renkema et al., 1998; Houston et al., 2003; Bussink et al., 2006). In contrast to both chitinases, chi-lectins lack the conserved additional chitin-binding domain (Boot et al. 1995; Renkema et al., 1997; Boot et al., 2001). The physiological function of the various chi-lectins is unknown. Similar to chitotriosidase and
AMCase, chi-lectins are secreted locally or into the circulation and a role in inflammatory conditions is suggested. For example, human cartilage GP39 (Hcgp39/YKL-40/CHI3L1), a protein expressed by chondrocytes and phagocytes has been implicated in arthritis, tissue-remodelling, fibrosis, cancer and asthma (Verheijden et al., 1997; Johansen, 2006). Similarly, the human chi-lectin YKL-39 (CHI3L2) and the murine chi-lectins Ym1 (Chi3L3/ECF-L) and Ym2 (Chi3L4) have been associated with the pathogenesis of arthritis (Hu et al., 1996; Tsuruha et al., 2002) and allergic airway inflammation, respectively (Chang et al., 2001; Ward et al., 2001; Homer et al., 2006; Webb et al., 2001). The high molecular weight oviductins, consisting of the amino-terminal 39 kDa catalytic domain followed by a heavily glycosylated Ser/Thr rich domain, are secreted by nonciliated oviductal epithelial cells and have been shown to play a role in fertilization and early embryo development (reviewed by Buhi, 2002).

Human chitotriosidase is exclusively expressed by phagocytes, namely in macrophages and neutrophils (Hollak et al., 1994; Escott and Adams, 1995). Tissue macrophages can massively express chitotriosidase as has for instance been demonstrated in Gaucher disease and atherosclerotic plaques (Renkema et al., 1995; Boot et al., 1999). Lysosomal stress is an important inducer of chitotriosidase in macrophages. By far the highest levels of chitotriosidase are found in Gaucher disease, but other diseases characterized by lysosomal accumulation of glycosphingolipids or other lipid species show increased levels as well. Examples of this are Niemann-Pick A/B, Niemann-Pick C, Krabbe, GM1 gangliosidosis, Cholesteryl ester storage disease, Wolman disease, Morquio B, and Tangier disease (Guo et al., 1995; Aerts et al., 2005). Elevated levels have also been found in fucosidosis, galactosialidosis, glycogen storage disease IV and Alagille syndrome (Michelakakis et al., 2004).

A common denominator for the induction of chitotriosidase in macrophages is presently not known. Possible candidates are gangliosides since secondary abnormalities in these lipids are commonly observed in lysosomal storage disorders (Walkley, 2004). In the case of Gaucher disease, markedly elevated concentrations of the ganglioside GM3 in tissues and plasma have also been recently reported (Ghauharali-van der Vlugt et al., 2008; Meikle et al., 2008). Considerable attention is presently focused to the ganglioside GM3, given its causal role in insulin resistance, the first abnormality in the development of type 2 diabetes. Recent literature links insulin resistance in tissues to the presence of excessive amounts of GM3. The ganglioside GM3 and other glycosphingolipids are found in specific (detergent resistant) membrane microdomains in close physical proximity to the insulin receptor (Inokuchi, 2006). A regulatory role for GM3 in insulin sensitivity is substantiated by a rapidly growing body of experimental evidence. Tagami and coworkers firstly demonstrated that addition of GM3 to cultured adipocytes suppresses phosphorylation of the insulin receptor and its down-stream substrate IRS-1, resulting in reduced glucose uptake (Tagami
et al., 2002). Inokuchi and coworkers reported that exposure of cultured adipocytes to TNF-alpha increases GM3 and inhibits IR and IRS-1 phosphorylation. This was found to be counteracted by 1-phenyl-2-decanoylamino-3-morpholinopropanol (PDMP), an inhibitor of glycosphingolipid biosynthesis (Kabayama et al., 2005). Mutant mice lacking GM3 have been reported to show an enhanced phosphorylation of the skeletal muscle insulin receptor after ligand binding and to be protected from high-fat diet induced insulin resistance (Yamashita et al., 2003). Consistent with this is the recent report on improved insulin sensitivity and glucose tolerance in mice with increased expression of the GM3 degrading sialidase Neu3 (Yoshizumu et al., 2007).

Conversely, GM3 levels are elevated in the muscle of certain obese, insulin resistant mouse and rat models (Aerts et al., 2007). Altered sphingolipid metabolism, reflected by increased glycosphingolipid, has also been recently documented in relation to neuronal pathology in diabetic retinopathy (Fox et al., 2006). Very recently Kabayama et al. provided evidence that the interaction of GM3 with the insulin receptor is mediated by a specific lysine residue located just above the transmembrane domain of the receptor, and that excess of GM3 promotes dissociation of the insulin receptor from caveolae, a location which is essential for signal transduction (Kabayama et al., 2007).

The value of pharmacological lowering of excessive ganglioside to improve insulin sensitivity has recently been demonstrated. Holland and coworkers reported that inhibition of the synthesis of ceramide, the precursor of glycosphingolipids, markedly improves glucose tolerance and prevents the onset of diabetes in obese rodents (Holland et al., 2007). Zhao et al. demonstrated that inhibition of the first step in biosynthesis of glycosphingolipids catalyzed by glucosylceramide synthase exerts beneficial effects. The inhibitor (1R,2R)-nonanoic acid[2-(2,3-dihydro-benz [1,4] dioxin-6-yl)-2-hydroxy-1-pyrrolidin-1-ylmethyl-ethyl]-amide-L-tartaric acid salt (Genz-123346) lowered glucose and HbA1C levels and improved glucose tolerance in insulin resistant rodents (Zhao et al., 2007). Finally, AMC researchers showed that treatment of various rodent models of insulin resistance with the aza-sugar N-(5-adamantane-1-yl-methoxy)-pentyl-1-deoxynojirimycin (AMP-DNM), a well tolerated potent inhibitor of glucosylceramide synthase, very markedly lowered circulating glucose levels, improved oral glucose tolerance, reduced HbA1C, and improved insulin sensitivity in muscle and liver (Aerts et al., 2007).

Entirely consistent with a role for the ganglioside GM3 in insulin resistance are recent findings with Gaucher patients. The increased concentrations of GM3 in plasma and tissue of Gaucher patients coincide with the occurrence of insulin resistance in these individuals (Ghauharali et al., 2008; Langeveld et al., 2008a; Langeveld et al., 2008b). Overt hyperglycaemia does however not occur in affected Gaucher patients, probably because of increased glucose consumption by the vast amounts of macrophages. Following rapid removal of storage macrophages by enzyme replacement therapy, overweight and type 2 diabetes
are more frequently encountered among Gaucher patients (Langeveld et al., 2008b). The final chapters of this thesis (addenda I and II) deal with gangliosides, insulin resistance and type 2 diabetes.

REFERENCES


Johansen, J.S. (2006). Studies on serum YKL-40 as a biomarker in diseases with inflammation, tissue...


OUTLINE OF THE THESIS

This thesis deals primarily with fundamental studies on the features and origin of mammalian chitinases.

Chapter 2 reviews in depth various facets of chitinase biology and biochemistry.

In Chapter 3, attention is focused to the physiological function of chitotriosidase. Its cellular origin and secretion in response to various biochemical stimuli are reported. Evidence from in vitro and in vivo studies is presented that substantiates a fungistatic role for chitotriosidase.

In Chapter 4, the common occurrence and consequence regarding enzymatic features of a G102S substitution in chitotriosidase is reported.

Chapter 5 deals with the molecular evolution of chitinases. Light is shed on the origin of the chitinase protein family with implications for chitinase-related biomedical research using model organisms.

Chapter 6 elaborates on species differences in chitinase expression between mouse and man. Tissue specific expression of chitinases has been studied in detail, revealing important differences that need to be taken into account when extrapolating results obtained in mice to human physiology.

Chapter 7 describes a novel method of measuring chitinase activity using chito-oligosaccharides as substrates. The improved assay offers far higher sensitivity than previously employed assays, facilitating chitinase detection and characterization.

In Chapter 8, structural features of the mammalian acidic chitinase, AMCase, are described. Modelling of the protein structure combined with molecular dynamic simulation and mutagenesis revealed the importance of subtle structural differences among chitinases.

Chapter 9 offers a comparison between enzymatic properties of chitinases of three species. Inter-species differences are described. Furthermore, it was assessed whether, due to a phenomenon known as transglycosylation, the proteins can be exploited for synthetic purposes.

Chapter 10 provides a summary and discussion of the various investigations on chitinases.
The thesis contains two additional chapters that are not related to chitinases, but concern other topics.

**Addendum 1** describes a novel method for visualizing gangliosides present on the cell surface.

**Addendum 2** deals with a post-translational protein modification, serine/threonine O-GlcNAcylation, that has been implicated in type II diabetes.