Origin and features of mammalian chitinases
Bussink, A.P.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
COMMON G102S POLYMORPHISM
IN CHITOTRIOSIDASE
DIFFERENTIALLY AFFECTS ACTIVITY
TOWARDS
4-METHYLMUMBELLIFERYL SUBSTRATES

Anton P. Bussink*, Marri Verhoek*, Jocelyne Vreede†,
Johannes M. F. G. Aerts*, and Rolf G. Boot*

† Department of Medical Biochemistry, Academic Medical Centre, University of Amsterdam,
Amsterdam, The Netherlands,
‡ Department of Internal Medicine, Academic Medical Centre, University of Amsterdam,
Amsterdam, The Netherlands,
† Van ’t Hoff Institute for Molecular Sciences, Universiteit van Amsterdam, Amsterdam, The
Netherlands

Submitted for Publication in revised form
ABSTRACT

Chitotriosidase (CHIT1) is a chitinase that is secreted by activated macrophages. Plasma chitotriosidase activity reflects the presence of lipid-laden macrophages in patients with Gaucher disease (GD). CHIT1 activity can be conveniently measured with fluorogenic 4-methylumbelliferyl (MU)-chitotrioside or 4MU-chitobioside as substrate, however non-saturating concentrations have to be used due to apparent substrate inhibition. Saturating substrate concentrations can however be used with the newly designed substrate 4-MU-deoxychitobioside. We studied the impact of a known polymorphism, G102S, on catalytic properties of CHIT1. The G102S allele was found to be common in Type 1 GD patients (~24% of alleles). The catalytic efficiency of recombinant Ser102 CHIT1 was ~70% of that of wild-type Gly102 CHIT1 when measured with 4MU-chitotrioside at non-saturating concentration. However, the activity was normal with 4MU-deoxychitobioside as substrate at saturating concentration, consistent with the prediction by molecular dynamics simulations. In conclusion, the interpretation of CHIT1 activity measurements with 4MU-chitotrioside with respect to CHIT1 protein concentrations depends on the presence of Ser102 CHIT1 in an individual, complicating estimation of body burden of storage macrophages. Use of the superior 4MU-deoxychitobioside substrate avoids such complications since the activity towards this substrate at saturating conditions is not affected by the G102S substitution.

INTRODUCTION

Gaucher disease (GD; MIM 230800), is a recessively inherited disease that is due to deficient activity of the lysosomal glucocerebrosidase (GBA; MIM 606463) [1]. Although glucocerebrosidase is present in lysosomes of all cell types, type I Gaucher disease (GD) patients exclusively develop storage of glucosylceramide (GL-1) in macrophages. It is believed that the storage material in macrophages stems from the breakdown of exogenous lipids derived from the turnover of blood cells. The characteristic lipid-laden macrophages, Gaucher cells, accumulate in the liver, spleen, and bone marrow. Gaucher disease is characterized by hepatosplenomegaly, haematological abnormalities, and skeletal involvement [1,2]. There is a remarkable spectrum of clinical severity among Type 1 GD patients. The limited correlation of genotype with phenotype stimulated a search for secondary biochemical markers that might indicate disease severity [for review, see ref. 3]. The importance of markers reflecting disease progression and correction further increased with the introduction of enzyme replacement therapy (ERT) [4] and substrate reduction therapy (SRT) [5,6]. Several serum abnormalities in GD patients have been documented (i.e., Macrophage colony-stimulating factor, angiotensin converting enzyme, tartrate-resistant acid
phosphatase, CD163, and CCL18), [7-9]. The most striking abnormality is elevated plasma chitotriosidase (CHIT1) activity [10]. CHIT1 is a chitinase secreted by alternatively activated human macrophages [11,12]. CHIT1 is produced as a 50 kDa protein, consisting of a chitin-binding domain, a hinge region and a 39 kDa catalytic domain in which the enzymatic activity resides [13]. The enzyme is secreted as 50 kDa protein into the circulation [14]. Plasma chitotriosidase activity is increased in several lysosomal [15-19] and nonlysosomal diseases [20]. In untreated GD patients, the median activity is about 600-fold greater than that in normal controls [10]. Plasma CHIT1 activity has proven useful for monitoring disease severity and the effectiveness of therapy in GD including ERT [21-25], and more recently, substrate reduction therapy [26,27]. In 2004, the International Collaborative Gaucher Group (ICGG) formally recommended plasma chitotriosidase activity as the biomarker of choice for evaluating GD patients and monitoring the effectiveness of ERT. Monitoring therapeutic response by measurement of plasma chitotriosidase activity suffers from two limitations. The assay of CHIT1 activity with commercially available substrates is complicated by the existence of apparent substrate inhibition due to transglycosidase activity [28]. Because of this, activity cannot be measured at saturating substrate concentrations and does not accurately reflect chitotriosidase protein levels. A novel substrate, 4MU-deoxychitobiose, has been developed that allows more accurate and sensitive measurement of chitotriosidase [28,29]. Another pitfall results from the complete absence of the enzymatic activity in about 6% of Caucasian individuals and even higher percentages in individuals of Asian ancestry [30-32]. This trait is caused by homozygosity for a 24-base pair duplication in exon 10, designated dup24, in the CHIT1 gene, preventing formation of active enzyme [30]. Plasma CHIT1 levels in heterozygotes for this null allele underestimate the actual presence of Gaucher cells in patients. Determination of CHIT1 genotype in Gaucher patients is therefore recommended. A further polymorphism resulting in a G102S substitution exists in the CHIT1 gene (MIM 800031). This was firstly reported by Gray and collaborators (patent application WO 97/47752). Coinciding with our investigation, Desnick and coworkers reported the common occurrence of the G102S allele among GD patients and normal subjects [31]. The Ser102 CHIT1 enzyme was found to show a reduced catalytic efficiency towards the artificial substrate 4MU-chitotrioside as compared to wild-type enzyme. We have investigated in detail the frequency of G102S CHIT1 allele and the impact of the amino acid substitution on catalytic efficiency towards various substrates. The interpretation of plasma chitotriosidase activities when measured with various substrates with respect to estimating disease severity is discussed.
MATERIALS AND METHODS

PATIENT SPECIMENS
Peripheral blood was collected from Type 1 GD patients and normal subjects evaluated at the Academic Medical Center. All specimens were obtained with informed consent and approval of the ethical review board of the institution. Baseline data on sex, age, splenectomy, severity score index and genotype were recorded. Volumes of liver were derived from MRI images as described earlier [24]. Excess liver volume was derived by subtracting a notional ‘expected’ liver volume (2.5% of body weight) from the observed liver volume.

PLASMA CHITOTRIOSIDASE ENZYME ASSAYS
Chitotriosidase activity in plasma samples, stored at -80 °C, was measured with the natural chitin fragment chitohexaose, or the fluorogenic substrates 4MU-chitotrioside, 4MU-chitobioside and 4MU-deoxychitobioside. Chitohexaose was obtained from Seikagaku Corporation, Tokyo, Japan, 4MU-chitotrioside and 4MU-chitobioside from Sigma, St Louis, MO. 4MU-deoxychitobiose was synthesized as earlier described [28]. Briefly, for the enzyme activity assay with 4-MU-substrates, 25 ml serum, diluted with BSA/PBS (bovine serum albumin/phosphate buffered saline, 1 mg/ml) and 100 ml substrate mixtures were incubated for 20 min at 37°C. For the determination of activity ratios, the substrate mixtures contained 0.0113 mM 4MU-chitotriose, or 0.027 mM 4MU-chitobiose or 0.250 mM 4MU-deoxychitobiose and 1 mg/ml BSA in McIlvain buffer, pH 5.2. Reactions were stopped with 2.0 ml 0.3 M glycine NaOH buffer pH 10.6 and the formed 4MU was detected fluorometrically (excitation at 366 nm; emission at 445 nm). Only less than 10% difference in the duplicates was allowed. One unit (U) of activity is defined as 1 nmol of substrate hydrolyzed per hr. Activity towards the natural oligosaccharide chitohexaose was measured using a HPLC method essentially as described previously [28]. Rather than relying on UV detection, sensitivity was increased by labelling of released fragments with fluorescent anthranilic acid (Bussink, submitted for publication).

IN GEL ENZYMATIC ASSAY
In gel chitinase activity was determined in a 12% polyacrylamide gel containing SDS, run in absence of β-mercaptoethanol. Renaturing of separated proteins was accomplished by incubating the gel for 16 hrs at room temperature in a casein-containing suspension (2.5 gr/L casein, 20 mM Tris, 2 mM EDTA, pH 8.5). Prior to exposure to artificial substrate the gel was washed three times in 30 mM NaAc/HAc (pH 5.2). The gel was soaked in 250 mM 4MU-deoxychitobiose for one minute, after which the fluorescent signal was determined at various exposure times in a Roche Lumi-Imager with settings optimized for 4MU fluorescence.
CH11 GENOTYPING
DNA was isolated from peripheral blood using the Gentra PureGene kit (Minneapolis, USA). Detection of the common dup24 insertion in exon 10 of the CH11 gene (NM_003465.1) was performed as described (30). The G102S mutation was detected by polymerase chain reaction amplification of the appropriate fragment (primers: RB203 5’-ggcagctggcagagtaaatcc-3’ & RB204 5’-cccagaaggaaattcagccc-3’) and sequencing (Big Dye Terminator sequencing kit, Applied Biosystems, according to manufacturers protocol on an Applied Biosystems 377A automated DNA sequencer).

ISOLATION AND EXPRESSION OF NORMAL AND MUTANT CHIT-1 DNA
CH11 cDNA was cloned previously (13). A fragment of the cDNA encoding the 39 kDa catalytic domain was used for recombinant protein production. The G102S point mutation was introduced directly into the wild-type CH11 cDNA in the expression plasmid, pcDNA3.1, using a fragment containing the G102S amplified from an individual that contained this polymorphism. Large-scale production and purification of the wild-type and mutant cDNA expression plasmids were performed using Qiagen Plasmid Midi Kits.

COS-7 cells were plated in complete media in six-well plates at a cell density of 1-3 x 10^5 cells per well and left overnight to achieve the desired cell concentration of 50 to 80% confluency. On the day of transfection, the complete media in each well was replaced with 1 mL of serum-free media. Transient transfection with the expression plasmid pcDNA3.1 containing the wild-type or mutant CH11 cDNA was achieved using FuGene 6 transfection reagent according to the manufacturer’s protocol (Roche Applied Science, Indianapolis, USA). After 72 hr, the media was collected and subjected to chitotriosidase assays.

WESTERN BLOT ANALYSIS
An antiserum raised against recombinant produced chitotriosidase [11] was used to visualize chitotriosidase protein on western blots. The presence of N-linked glycans was determined by monitoring the shift in molecular mass of chitotriosidase upon digestion with endoglycosidase F (PNGase F, New England Biolabs).

DETERMINATION OF SPECIFIC ACTIVITY OF NORMAL AND SER102 CH11
The specific activity of recombinantly produced wild-type and Ser102 CH11 was comparatively assessed by comparison of intensity of cross reactive material with western blot analysis using a similar input of enzymatic activity of both enzymes. Quantification of cross reactive material was performed using Quantity One analysis software (Bio-Rad laboratories). For comparison, a pure standard of recombinant chitotriosidase, previously produced enzyme at large scale and for which specific activity had been determined by protein measurement [33]. The
specific activity of plasma wild-type and Ser102 CHIT1 was also determined using label-free LC-MS as recently described [34]. Plasma was analyzed from an individual expressing both G102S and wild-type chitotriosidase, and an individual expressing only wild-type enzyme.

**Modelling of the G102 Substitution and Molecular Dynamics Simulation**

The model of Ser102 CHIT1 was based on the crystal structure of native chitotriosidase (Research Collaboratory for Structural Bioinformatics (RCSB) protein data base (PDB) acc. nr: 1LQ0, resolution 2.20 Å) [35]. The glycine at position 102 was converted into a serine using the program Deepview [36]. Both the native and modified structure were subjected to energy minimalization in GROMACS version 3.3.1 with the GROMOS96 forcefield using the steepest decent method [van Gunsteren, W. F., and H. J. C. Berendsen. 1987. GROMOS-87 Manual. BIOMOS BV, Groningen, The Netherlands]. Preparation of the systems for molecular dynamics (MD) included solvation of the protein structure in a periodic, cubic box, addition of polar and aromatic hydrogen atoms (at a pH of 5.2), addition of SPC water molecules [37], removal of water molecules residing in hydrophobic cavities and charge neutralization by exchanging waters with chloride ions. Prior to actual MD the systems were subjected to another round of energy minimalization, followed by 20 ps of MD with position restraints on heavy protein atoms and an unconstrained equilibration run of 1 ns. Both temperature and pressure in the systems was kept constant, at 300 K and 1 bar, respectively, using the Berendsen-thermostat and -barostat. Bonded interactions were described with the GROMOS96 forcefield, van der Waals interactions and short range electrostatic interactions were treated with a cut-off radius of 1.0 nm and long-range electrostatic interactions were treated with the particle mesh Ewald method [38]. Using the LINCS algorithm to constrain bonds [39] allowed for a timestep of 2 fs. Prepared as such, the dynamics of the two systems were sampled during three separate MD runs of 10 ns, initiated from different starting velocities. From the resulting trajectories Root Mean Square Fluctuations (RMSF) were calculated using the tools included in the GROMACS software package.

**Statistical Analysis**

The data were analyzed using the Mann-Whitney U test. Correlations were tested by the rank correlation test (Spearman coefficient, ρ). P values less than 0.05 were considered statistically significant.

**Results**

**Frequency of CHIT1dup24 and CHIT G102S**

The CHIT1 genotype was determined in a large number of Gaucher patients of European ancestry (n=86). Among the Gaucher patients, 3.5% and 41.7% were
homozygous or heterozygous, respectively, for the G102S mutation, with an allele frequency of 0.24 (41/172). Among the same patients, 6% and 27% were homozygous or heterozygous for the dup24 allele respectively, with an allele frequency of 0.20 (35/172). The numbers of detected homozygotes for the G102 allele and the dup24 allele were consistent with the Hardy Weinberg equilibrium. Sequencing the CHIT1 gene of selected cases revealed that in the GD patient cohort all four conceivable CHIT alleles occurred (allele containing duplication without G102S mutation, allele containing duplication with G102S mutation, allele without duplication and without G102S mutation, allele without duplication and with G102S mutation).

**Enzymatic activity of chitotriosidase towards various artificial substrates**

Chitotriosidase activity in plasma samples of 47 type 1 Gaucher patients with an established CHIT genotype was measured using 4MU-chitotrioside and 4MU-deoxychitobiose as substrates. A significant correlation between G102S genotype and the activity towards the two artificial substrates became apparent when analyzing the results for individuals lacking the dup24 allele (figure 1). Individuals that solely express the wild-type Gly102 enzyme (genotype G/G) display the highest 4MU-chitotrioside/4MU-deoxychitobiose (trio/deoxybio) activity ratios, while individuals that express solely the Ser102 enzyme (genotype A/A) have substantially lower trio/deoxybio activity ratios. The heterozygotes (genotype G/A) show intermediate values.

![Figure 1](image-url)
In the case of carriers of the dup24 allele a broad range of reduced trio/deoxybio activity ratios was observed (not shown). As established by sequencing of large segments of CHIT1 genes, this is explained by the fact that in some individuals the G102S mutation is on the same allele as the duplication and only wild-type protein is produced, whilst in others the G102S mutation is on the wild-type allele and G102S substituted enzyme is solely present.

Next, the activity of recombinant produced 39 kDa wild-type and Ser102 CHIT1 towards 4MU-chitotrioside and 4MU-deoxychitobiose was determined. Recombinant Ser102 CHIT1 showed a clearly reduced (75% of wild type enzyme) trio/deoxybio activity ratio, mimicking the findings made with plasma enzymes. This suggests that the catalytic efficiency of Ser102 CHIT1 towards 4MU-deoxychitobiose is normal, but slightly impaired towards 4MU-chitotrioside. Of note, enzyme activity measurement with the substrate 4MU-chitobioside revealed that the G102S substitution, either in plasma enzyme or recombinant chitotriosidase, did not affect markedly the bio/deoxybio activity ratio (not shown).

**GLYCOSYLATION OF G102S CHITOTRIOSIDASE**

The G102S mutation creates a potential glycosylation site at Asp100 within the 39 kDa catalytic domain of chitotriosidase. To test the possibility that the mutant enzyme is indeed glycosylated, we compared recombinant produced 39 kDa wild-type and Ser102 CHIT1 using western blot analysis. As can be seen from figure 2A the mutant enzyme shows an additional, less intense, cross-reactive protein with a slightly higher molecular mass than 39 kDa. To assess the nature of this additional isoform, we subjected the recombinant proteins to endoglycosidase F digestion. Figure 2B shows that the additional isoform of the mutant enzyme is sensitive to the endoglycosidase F digestion, suggesting that it is glycosylated. Following electrophoretic protein separation in a SDS-acrylamide gel, two isoforms could also be visualized by detecting hydrolysis of the fluorogenic 4MU-deoxychitobiose substrate (figure 2C, upper panel). Apparently, both isoforms are enzymatically active.

Next, plasma samples of Gaucher patients with different genotypes (G/G, G/A and A/A), and lacking the dup24 allele, were subjected to western blot analysis. Figure 2D shows that in the case of plasma from patients that strictly express the wild-type enzyme of 50 kDa, only a single cross-reactive band is detected. However, samples from patients that carry the mutant allele display an additional cross-reactive band above the 50 kDa protein, that was found to be sensitive to endoglycosidase F digestion (not shown). The additional band is more intense in the case of homozygotes for Ser102 CHIT1 (A/A) than heterozygotes (G/A) (figure 2D).

**SPECIFIC ACTIVITY OF WILD-TYPE AND SER102 CHIT1**

To determine whether the G102S substitution in CHIT1 affects catalytic efficiency towards 4MU-chitotrioside and 4MU-deoxychitobiose, the specific activity of
COS-produced recombinant wild-type and Ser102 CHIT1 was studied. Unfortunately, an accurate direct measurement of protein concentration was not feasible given the low quantities of recombinant enzymes available. Using SDS-PAGE and western blotting, the catalytic efficiency of 39 kDa wild-type and Ser102 CHIT1 was compared (figure 3). Applying an equal amount of activity towards 4MU-deoxychitobioside for both enzymes, resulted in equally intense amounts of cross reactive material. However, applying an equal amount of activity towards 4MU-chitotrioside for both enzymes, resulted in less cross reactive material in the case of wild-type enzyme (60-80% compared to mutant enzyme). Thus, the specific activity of Ser102 CHIT1 towards 4MU-chitotrioside appears to be reduced. Recently, a label-free LC-MS method was developed that allows absolute quantification of CHIT1 protein in plasma specimens [34]. CHIT1 protein concentrations in plasma samples were measured in both a heterozygous individual and a homozygous wild type individual. The specific activity towards
4MU-chitotrioside was lowest in the case of the plasma sample containing both enzymes (3.25 mmol/mg.hour), and highest in plasma containing only wild-type CHIT1 (4.09 mmol/mg.hour). This confirms the observations (figure 3) that Ser102 CHIT1 is only slightly impaired in activity towards 4MU-chitotrioside.

Other enzymatic features of wild-type and Ser102 CHIT1 were comparatively investigated. Both enzymes showed apparent substrate inhibition with 4MU-chitotrioside as substrate, a phenomenon that is caused by transglycosylation of this substrate (not shown). Fortunately, the substrate 4MU-deoxychitobioside can not be transglycosylated and shows Michaelis-Menten kinetics allowing determination of Km. The Km of Ser102 CHIT1 for the 4MU-deoxychitobioside (determined by means of Eadie–Hofstee plotting and linear regression) is 102 +/- 6 μM, substantially higher than that of wild type enzyme (43 +/- 1 mM). Both recombinant proteins were found to be active towards the natural chito-oligomer chitohexaose releasing both chitobiose and chitotriose moieties from the chitohexaose (Table 1). The ability of G102S chitotriosidase to hydrolyze this natural chitin oligomer appeared only marginally reduced compared to wild-type enzyme.

**Figure 3.** Apparent specific activity of recombinant wild-type and Ser102 CHIT1. Equal amounts of activity of recombinant wild-type and Ser102 CHIT1, either with 4MU-chitotrioside (left) or 4MU-deoxychitobioside (right) were subjected to Western blot analysis and quantified as described in the experimental procedures.

**Table 1.** Formation of fragments from chitohexaose (expressed in μM) by wild-type and Ser102 CHIT1.

<table>
<thead>
<tr>
<th>Substrate (GlcNAc)</th>
<th>60 μM</th>
<th>120 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNAc2</td>
<td>23.6</td>
<td>19.7</td>
</tr>
<tr>
<td>GlcNAc3</td>
<td>27.7</td>
<td>20.0</td>
</tr>
<tr>
<td>GlcNAc4</td>
<td>13.3</td>
<td>13.5</td>
</tr>
<tr>
<td>GlcNAc6</td>
<td>33.0</td>
<td>90.0</td>
</tr>
</tbody>
</table>

**Modelling of the G102S substitution**

The three-dimensional structure of CHIT1 has been extensively studied by crystallography [35,40] and therefore a reliable prediction can be made for the enzyme structure containing a serine instead of glycine at amino acid position 102. The protein was shown to adopt a highly stabilized (β/α)8-fold, also known as TIM barrel. Mutation of the glycine into a serine did not alter the overall structure, as can be concluded from the near superimposability of the energy minimalized structures of the 102G and 102S proteins (r.m.s. deviation 0.02 Å). However, since Ser102 is located close to the binding cleft, we investigated whether possible
hydrogen-bonding interactions of the serine hydroxyl could result in altered substrate-binding. Since the G102S mutation was shown to effect hydrolysis of the chitotrioside-substrate and to a lesser extent the chitobioside-substrate, it was hypothesized that differences in binding of the third sugar (at the -3 position) are responsible for the observed differences in activity. Therefore, the published crystal structure of the chitotriosidase-allosamidin complex was carefully examined [40]. Allosamidin is a potent chitinase inhibitor consisting of two N-acetylglucosamine residues and a group that mimics the transition-state analogue and therefore can be used to assess positioning of the second and third sugar residues in the binding cleft. The structure indeed reveals a hydrogen bonding interaction between the N-acetyl moiety of the third sugar and Asn100.

In order to evaluate differences between both proteins we performed molecular dynamics (MD), simulations in which atoms are allowed to interact for a period of time under known laws of physics, providing insight in the motion of atoms. Simulations of both native wild-type and mutant unglycosylated structures were performed. The 10 ns MD runs show a considerable overall rigidity of secondary structures, as shown by r.m.s. fluctuations (RMSF, a measure for flexibility) of 0.05-0.15 nm, consistent with the compact, highly stabilized structure of the $(\beta/\alpha)_8$ barrel. Furthermore, the catalytic glutamic acid is accessible to solvent, compatible with hydrolase activity. Comparison of the residue-specific RMSF between wild-type and G102S chitotriosidase shows a markedly decreased mobility for residues 96-104 in the G102S protein, corresponding to the loop separating $\beta_3$ and $\alpha_3$ containing Asn100 (figure 4A). Visual inspection of the MD trajectories indeed shows the hydroxyl oxygen of Ser102 to be able to form additional hydrogen bonds with the peptide backbone at Phe101 and Lys105 and the side chain of both Gln104 and Lys105, resulting in a demobilization of the loop (figure 4B). Since the G102S substitution results in a marked decrease in flexibility it is conceivable that the sugar at the -3 position can no longer be stabilized by Asn100, which is likely to result in a lower activity of the enzyme towards the 4MU-chitotrioside substrate. It presently remains, however, unclear how the mutation affects the binding constant of the enzyme for 4MU-deoxychitobioside.

**Correlation of wild-type and Ser102 CHIT1 with severity of Gaucher disease manifestation**

CHIT1 is a useful biomarker to estimate disease severity and to monitor the effectiveness of ERT. Since CHIT1 is secreted from pathological lipid laden Gaucher cells that predominantly accumulate in liver, spleen and bone marrow, a correlation between enzyme activity and both excess-liver and -spleen volume was proposed and indeed demonstrated [25]. The findings presented above show that the G102S substitution results in an underestimation of the amount of CHIT1 protein when measured enzymatically with 4MU-chitotrioside. In light of this, we
examined for a cohort of type 1 GD patients lacking the dup24 allele and with an intact spleen, the correlation of excess liver volume and plasma CHIT1 employing both 4MU-chitotrioside and 4MU-deoxychitobiose as substrates. We observed a $\rho$ of 0.58 ($P=0.0004$) when plasma chitotriosidase activities were measured with 4MU-chitotrioside. Using 4MU-deoxychitobiose for activity measurements, statistical significance increased to 0.66 ($P<0.0001$). Thus, the correlation between excess liver volume and CHIT activity indeed improves when using 4MU-deoxychitobiose as substrate for enzyme measurements.

**Figure 4.** Structural implications of the G102S mutation. A: R.m.s. fluctuations (RMSF) in the effected domain (residue numbers are shown on the x-axis, RMSF in nm on the y-axis) in wild-type (in grey) and Ser102 CHIT1 (in black). The values represent averages obtained from three independent runs. B: Superposition of wild-type and mutant structures (grey is wild-type). Amino acids 70-95 of both enzymes are coloured according to RMSF on a scale from blue (RMSF = 0 nm) to red (RMSF = 0.25 nm). The location of the mutation is highlighted (green in wild-type protein, yellow in Ser102 CHIT1). Sidechains of Ser102 and Lys105 having hydrogen bonded interactions are shown.
DISCUSSION

Coinciding with our investigation, the research groups of Desnick and Beutler independently characterized CHIT1 genotypes in various groups of individuals [31,32]. Like us, Desnick and coworkers noted in their study the common occurrence among GD patients and normal subjects of the dup24 and G102S alleles [31]. Interestingly, the observed frequency of the G102S allele was about 0.3 in subjects of various ancestries, including African. This is in sharp contrast to the situation for the dup24 allele that is far less frequent among individuals of African extraction as compared to subjects of European ancestry [32,41,42]. Concomitantly, Beutler and coworkers determined in an impressive series of individuals the frequency of the dup24 allele, being 0.56 (n=2054) in subjects of Asian ancestry, 0.17 (n=984) in subjects of European ancestry and 0.07 (n=536) in subjects of African ancestry [32]. They also reported high G102S allele frequencies for various ethnic groups, being 0.27, 0.26, and 0.24 for European (n=180), African (n=150) and Asian (n=904) groups, respectively. The results of our study with Gaucher patients of European ancestry are remarkably consistent to the reports by the groups of Desnick and Beutler. The frequency of the G102S allele in the patient population studied by us was 0.24 and that of the dup24 allele was 0.20. Of note, we observed that the 24 bp duplication and G102S mutation are not strictly linked and that all possible combinations of the CHIT1 alleles occur. It thus seems most likely that the two mutations in CHIT1 are ancient and that already among the founders of non-African ethnic groups carriers of all four different CHIT1 alleles must have existed.

The consequences of the G102S substitution in CHIT1 for its enzymatic efficiency are of interest. Desnick and coworkers reported a markedly (about 4-fold) reduced catalytic activity of Ser102 CHIT1 towards the artificial substrates 4MU-chitotrioside [31]. In contrast, Beutler and collaborators found no indications for significantly reduced activity of Ser102 CHIT1 [32]. In our hands, the specific activity of recombinant Ser102 CHIT1 towards 4MU-chitotrioside is about 70 % of normal. A similar extent of reduction in specific activity was noted for plasma-derived Ser102 CHIT1. Desnick and coworkers compared the specific activity of wild-type and Ser102 CHIT in media of COS-transfected cells using silver-staining after gel electrophoresis. In their case, the protein signal staining intensities of aliquots containing almost equal 4MU-chitrioside hydrolyzing activity were much higher in the case of Ser102 CHIT1 than wild-type enzyme. It was concluded that Ser102 CHIT1 only showed 23% of wild-type catalytic activity. In our hands, the differences between wild-type and Ser102 CHIT1 in activity towards 4MU-chitotrioside are much smaller. A possible, quite trivial explanation for the apparent differences in findings among various research groups may be that very low substrate 4MU-chitotrioside concentrations have to be used in assays of CHIT1 activity. The binding constant of Ser102 CHIT1 for this substrate could very well...
differ from that of wild-type Gly102 enzyme. Unfortunately, this constant can not be experimentally determined due to the ongoing transglycosylation of the 4MU-chitotrioside substrate [28]. However, using 4MU-deoxychitobioside as substrate, which can not undergo transglycosylation, a substantially higher Km in the case of Ser102 CHIT1 was observed by us. It is therefore conceivable that the binding constant for 4MU-chitotrioside is indeed effected by the G102S substitution in CHIT1 and that, in combination to this, slight differences in assay concentration of 4MU-chitotrioside among research groups might generate different results for relative specific activity of Ser102 CHIT1. Our finding that the G102S substitution has only a very small effect on hydrolysis of the natural chito-oligosaccharide chitohexaose indicates that Ser102 CHIT1 is not intrinsically impaired in hydrolytic activity. The same is suggested by the normal activity of the enzyme towards 4MU-deoxychitobioside. Structural modelling of the mutation combined with MD simulations offers an explanation for the observed difference in activity towards 4MU-chitotrioside. A decrease in flexibility was observed locally in the Ser102 protein, which is likely to result in slightly altered substrate binding. The consequence of the partial glycosylation of Ser102 CHIT1 is still unclear. Our investigation did not point to a major difference in enzymatic activity of glycosylated and unglycosylated enzyme when measured with 4MU-deoxychitobioside as substrate. Obviously, it can not be excluded that the glycosylated isoform is more rapidly (lectin-mediated) cleared from the circulation. Given the current application of plasma CHIT1 as measure for the body burden of Gaucher cells in GD patients and its use to assess disease severity and efficacy of therapeutic intervention, the genetic heterogeneity in the CHIT1 gene is of importance. This has been elegantly pointed out by Desnick and coworkers [31]. Interpretation of plasma CHIT1 activities, especially when determined with 4MU-chitotrioside as substrate, should take into account the CHIT1 genotype of an individual. Importantly, the newly developed substrate 4MU-deoxychitobiose offers a convenient solution. The catalytic efficacy towards this substrate seems not affected by the G102S substitution. The fact that 4MU-deoxychitobiose can not serve as acceptor in transglycosylation offers further advantages such as the use of saturating substrate concentration. Correction of measured plasma CHIT1 for patients carrying the G102S allele may be considered. According to the observed reduction in specific activity of Ser102 CHIT1, correction would imply multiplying measured levels of plasma chitotriosidase activity measured with 4MU-chitotrioside at our assay conditions with a factor of 1.3 in the case of carriers for the G102S allele and by a factor of 1.6 in the case of homozygotes for G102S allele. Applying such correction to our dataset improved the correlation between excess liver volume and plasma chitotriosidase in Gaucher patients: uncorrected \( \rho = 0.58 \) (P=0.0004), corrected \( \rho = 0.65 \) (P=0.0001), the latter almost identical to the \( \rho = 0.66 \) (P<0.0001) observed for chitotriosidase data obtained with 4MU-deoxychitobiose as substrate.
Obviously, it should be realized that the correction factor may differ between research groups, being highly dependent on the precise assay conditions, in particular 4MU-chitotrioside concentration. Moreover, it should be kept in mind that, although appealing, such correction is not feasible in carriers of both the G102S allele and dup24 allele. In such cases it is not known a priori whether the two mutations are at the same or distinct CHIT alleles. In the former situation no correction should be made and in the latter the correction should be by a factor of 1.6 when using our assay conditions.

In conclusion, the G102S substitution in CHIT1 occurs commonly among individuals of European ancestry, including Gaucher patients. Since this substitution negatively affects activity of CHIT1 towards 4MU-chitotrioside, measured plasma enzyme activities with this substrate may in some individuals insufficiently reflect chitotriosidase protein, the latter being related to the presence of storage cells. This could result in an underestimation of disease severity. For an optimal interpretation of plasma chitotriosidase activities in relation to monitoring disease severity, the use of the 4MU-deoxychitobioside substrate has to be therefore strongly recommended.

ACKNOWLEDGEMENTS

We gratefully acknowledge SARA Computing and Networking Services for allowing use of the LISA cluster and their skilful technical assistance. We acknowledge our clinical colleagues Maaike Wiersma, Mirjam Langeveld, Mario Maas and Maaike de Fost for collection of patient materials and records.

REFERENCES

biosynthesis. Lancet 355, 1481-1485


Vedder, A. C., Cox-Brinkman, J., Hollak, C. E., Linthorst, G. E., Groener, J. E., Helmond, M.


