TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. A I OSA - TOM. 433

ASTRONOMICA - CHEMICA - PHYSICA - MATHEMATICA

Characterisation of Human Neutral and Lysosomal α-Mannosidases

by

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Turku 2011

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ISBN 978-951-29-4849-9 (PRINT)
ISBN 978-951-29-4850-5 (PDF)
ISSN 0082-7002
Painosalama Oy – Turku, Finland 2011

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ABSTRACT

Neutral α-mannosidase and lysosomal MAN2B1 α-mannosidase belong to glycoside hydrolase family 38, which contains essential enzymes required for the modification and catabolism of asparagine-linked glycans on proteins. MAN2B1 catalyses lysosomal glycan degradation, while neutral α-mannosidase is most likely involved in the catabolism of cytosolic free oligosaccharides. These mannose containing saccharides are generated during glycosylation or released from misfolded glycoproteins, which are detected by quality control in the endoplasmic reticulum.

To characterise the biological function of human neutral α-mannosidase, I cloned the α-mannosidase cDNA and recombinantly expressed the enzyme. The purified enzyme trimmed the putative natural substrate $Man_9GlcNAc$ to $Man_5GlcNAc$, whereas the reducing end $GlcNAc_2$ limited trimming to $Man_8GlcNAc_2$. Neutral α-mannosidase showed highest enzyme activity at neutral pH and was activated by the cations Fe^{2+}, Co^{2+} and Mn^{2+}, Cu^{2+} in turn had a strong inhibitory effect on α-mannosidase activity. Analysis of its intracellular localisation revealed that neutral α-mannosidase is cytosolic and colocalises with proteasomes. Further work showed that the overexpression of neutral α-mannosidase affected the cytosolic free oligosaccharide content and led to enhanced endoplasmic reticulum associated degradation and underglycosylation of secreted proteins.

The second part of the study focused on MAN2B1 and the inherited lysosomal storage disorder α-mannosidosis. In this disorder, deficient MAN2B1 activity is associated with mutations in the *MAN2B1* gene. The thesis reports the molecular consequences of 35 α-mannosidosis associated mutations, including 29 novel missense mutations. According to experimental analyses, the mutations fall into four groups: Mutations, which prevent transport to lysosomes are accompanied with a lack of proteolytic processing of the enzyme (groups 1 and 3). Although the rest of the mutations (groups 2 and 4) allow transport to lysosomes, the mutated proteins are less efficiently processed to their mature form than is wild type MAN2B1. Analysis of the effect of the mutations on the model structure of human lysosomal α-mannosidase provides insights on their structural consequences. Mutations, which affect amino acids important for folding (prolines, glycines, cysteines) or domain interface interactions (arginines), arrest the enzyme in the endoplasmic reticulum. Surface mutations and changes, which do not drastically alter residue volume, are tolerated better. Descriptions of the mutations and clinical data are compiled in an α-mannosidosis database, which will be available for the scientific community.

This thesis provides a detailed insight into two ubiquitous human α-mannosidases. It demonstrates that neutral α-mannosidase is involved in the degradation of cytosolic oligosaccharides and suggests that the regulation of this α-mannosidase is important for maintaining the cellular homeostasis of N-glycosylation and glycan degradation. The study on α-mannosidosis associated mutations identifies multiple mechanisms for how these mutations are detrimental for MAN2B1 activity. The α-mannosidosis database will benefit both clinicians and scientific research on lysosomal α-mannosidosis.

TABLE OF CONTENTS

ABBREVIATIONS

cDNA	Complementary DNA
CHO-KI	Chinese hamster ovary cell line
COS-7	African green monkey kidney cells
DLO	Dolichol-linked oligosaccharide
Dol	Dolichol
dpm	Disintegrations per minute
EC	Enzyme commission number
EDEM	Endoplasmic reticulum degradation-enhancing α-mannosidase like protein
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescence protein
EGFP-kdel	Enhanced green fluorescence protein fusion with amino acids KDEL
EGFP-rab7	Enhanced green fluorescence protein fusion with lysosomal/ endosomal rab7 protein
ENGase	Endo-β-N-acetylglucosaminidase
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
ERmanI	Endoplasmic reticulum mannosidase I
FRAP	Fluorescence recovery after photobleaching
Free OS	Free oligosaccharides
GDP-Man	Guanosine diphosphate mannose
GH38	Glycoside hydrolase family 38
GH47	Glycoside hydrolase family 47
Glc	Glucose
GlcNAc	N-acetylglucosamine
GT-EGFP	The type II membrane-anchored protein galactosyltransferase fusioned with Enhanced green fluorescence protein
GIIAM	Golgi II α-mannosidase
GIIxAM	Golgi IIx α-mannosidase
HeLa	Human cervical carcinoma cells
HeLa-NAM	HeLa cell line overexpressing human neutral α-mannosidase
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
kDa	Kilodalton
LAMP1	Lysosome associated membrane protein 1
LMP2-GFP	Proteasome subunit LMP2 fusion with a green fluorescent protein
LSDs	Lysosomal storage disorders
Man	Mannose
MAN2B1	Lysosomal α-mannosidase
MAN2B2	Narrow-specificity lysosomal α-mannosidase
MAN2C1	Neutral α-mannosidase, NAM
MES	2-(N-morpholino) ethanesulfonic acid
M6P	mannose-6-phosphate

M8B	$Man_8GlcNAc_2$ isoform
M8C	$Man_8GlcNAc_2$ isoform
NAM	Neutral α-mannosidase, NAM
NAM-EGFP	Neutral α-mannosidase fusioned with Enhanced green fluorescence protein
N-glycan	Asparagine-linked glycan
NHK	Null Hong Kong variant of α-antitrypsin
OMIM	Online Mendelian inheritance in man
OS-P	Phosphorylated free oligosaccharides
OST	Oligosaccharyl transferase
P	Phosphate
PCR	Polymerase chain reaction
PDB ID	Protein data bank identification code
PDI	Protein disulfide isomerase
PNGase	Peptide:N-glycanase
PNP α-man	Para-nitrophenyl α-D-mannoside
R_{obs}	Pearson correlation coefficient, observed
R_{rand}	Pearson correlation coefficient, random
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interfering ribonucleic acid
SNP	Single nucleotide polymorphism
TEV	Tobacco etch virus protease
UDP-GlcNAc	Uridine diphosphate N-acetyl glucosamine
UGGT	Uridine diphosphate glucose:glycoprotein glucosyltransferase
3D	Three dimensional
6xHis	Six-histidine tag

Abbreviations for high mannose-type oligosaccharides are specified in Figure 1.

ORIGINAL PUBLICATIONS

This thesis comprises the following publications. I have referred them in the text with their roman numerals.

I Kuokkanen, E., Smith, W., Mäkinen, M., Tuominen, H., Puhka, M., Jokitalo, E., Duvet, S., Berg, T. and Heikinheimo, P. (2007). Characterization and subcellular localization of human neutral class II α-mannosidase. *Glycobiology* **17**, 1084-1093 Erratum in: *Glycobiology*, 2008, **18**, 136.

II Bernon, C., Carré, Y., Kuokkanen, E., Slomianny, M.C., Mir, A.M., Krzewinski, F., Cacan, R., Heikinheimo, P., Morelle, W., Michalski, J.C., Foulquier, F. and Duvet, S. (2011) Overexpression of Man2C1 leads to protein underglycosylation and upregulation of endoplasmic reticulum-associated pathway. *Glycobiology* **21**, 363-375.

III Kuokkanen, E., Riise Stensland, H.M., Smith, W., Kjeldsen Buvang, E., Nguyen, L.V., Nilssen, Ø. and Heikinheimo, P. (2011) Molecular and cellular characterization of novel α-mannosidosis mutations. *Hum. Mol. Genet.* **20**, 2651-2661.

IV Nilssen, Ø., Frantzen, G., Kuokkanen, E., Heikinheimo, P., Malm, D. and Riise Stensland, H.M. "amamutdb.no: A relational database for *MAN2B1* allelic variants which compiles genotypes, clinical phenotypes, and biochemical and structural data of mutant MAN2B1 in α-mannosidosis". *Manuscript*.

1. INTRODUCTION

Neutral α-mannosidase (NAM or MAN2C1, Enzyme commission number (EC) 3.2.1.24) and lysosomal α-mannosidase (MAN2B1: EC 3.2.1.24) are ubiquitous mammalian enzymes. In a sequence-based classification, they belong to glycoside hydrolase 38 family (GH38) together with three other mammalian α-mannosidases (1), (http://www.cazy.org/Glycoside-Hydrolases.html). These enzymes cleave the α-configurated mannosidic linkages, which are common in the asparagine-linked glycan (N-glycan) moieties on proteins. The mammalian GH38 α-mannosidases participate both in the biosynthesis and degradation of N-glycans. (2, 3).

N-glycosylation is an abundant post-translational modification found on secretory pathway proteins (4). It initiates as a co-translational event in the endoplasmic reticulum (ER) by the attachment of an oligosaccharide precursor onto the nascent peptide (5-7). N-glycans are already modified in the ER in a process which is important for the folding and transport of the successfully folded proteins into the Golgi (4, 8). The trimming of mannose residues has a central role when the misfolded proteins are recognised and directed to the cytosol by ER-associated degradation (ERAD), a pathway leading to proteasomal degradation of the peptide (8, 9). The N-glycan moieties from the ERAD-substrates are released into the cytosol as free oligosaccharides (free OS), and their cytosolic degradation has been suggested to involve NAM (10, 11). When transported into the lysosome, the trimmed free OS are suitable substrates for MAN2B1 (12, 13).

Although NAM is a ubiquitous enzyme and putatively important for N-glycan metabolism, its role in the cell is incompletely understood. In addition to the cytosol, NAM has also been purified from the ER-fraction of the cell (14-16). Down-regulation of NAM expression in human cancer cells was accompanied with modifications on extracellular N-glycans, and the NAM silenced cells became less malignant (17, 18). The extracellular consequences of silencing a cytosolic enzyme are challenging to explain, since the N-glycan structures on secreted proteins mainly result from the action of the enzymes of the secretory pathway (19).

MAN2B1 is involved in the lysosomal hydrolysis of α-mannosidic linkages (13, 20, 21). The enzyme is synthesised in the ER and transported via the Golgi to lysosomes (21, 22), where MAN2B1 is homodimeric and proteolytically processed at specific sites (22-24). The three dimensional (3D) structure for bovine MAN2B1, which has 80% sequence identity with human MAN2B1, has been solved (24). The lack of MAN2B1 enzyme activity is characteristic of α-mannosidosis (Online Mendelian Inheritance in Man, OMIM #248500), a rare inherited disease, which belongs to a group of lysosomal storage disorders (LSDs). In LSDs, lysosomal metabolism is affected due to mutations in the genes encoding lysosomal enzymes or enzymes related to lysosomal functions (25, 26). The age of onset, symptoms and their severity in α-mannosidosis are diverse, and a genotype – phenotype correlation has been difficult to identify. The present treatment only alleviates the symptoms and does not cure the disease. (27). Over 120 α-mannosidosis causing mutations have been identified (28), but only a minority of them has been characterised at the cell biological or molecular level (22, 29, 30).

2. LITERATURE REVIEW

2.1 Protein N-glycosylation and folding of the secretory pathway glycoproteins

In the first step of N-glycosylation, oligosaccharyl transferase (OST) transfers the preassembled oligosaccharide to the glycosylation sequence Asn-X-Ser/Thr (X represents any amino acid except proline) from a dolichol-linked oligosaccharide (DLO) (4, 6). The structure of the preassembled oligosaccharide is extensively conserved in eukaryotes (31): it is a branched-glycan with three glucose (Glc), nine mannose (Man) and two N-acetyl glucosamine (GlcNAc) residues (Figure 1). The glycans are trimmed of their three glucose residues and one mannose residue during protein folding in the ER (8). In the second step of N-glycosylation, the successfully folded proteins enter the Golgi, where glycosyl transferases and hydrolases further modify the oligosaccharides into complex type glycan structures (19). This process involves the breakdown of glycan to $Man_3GlcNAc_2$ and the rebuilding of the final molecule by the addition of monosaccharide residues in a prescribed manner, which demands several enzymes and energy (4, 31). Selected N-glycans avoid further trimming because they have gained a phosphorylation tag for lysosomal transport (32) or are embedded in the folded structure (24). These N-glycans remain as high mannose type oligosaccharides.

The conserved precursor N-glycan serves different functions in the ER than do the diverse mature N-glycans after the Golgi. The N-glycans have an impact on protein folding in the ER both due to their general physicochemical properties (33), and the specific signalling effects through various lectins (34), proteins which are able to recognise and bind carbohydrates. Lectin interactions are significant in transport to the Golgi, folding and quality control, which distinguishes folding-defective structures from native ones and delivers permanently misfolded proteins for degradation (9, 34). Lectin recognition involves specific residues, thus it is necessary that proteins passing through the ER display these specific carbohydrate tags. After maturation in the Golgi, the N-glycans have diversified their structures in respect to the composition of their monosaccharides, oligosaccharide chain length and branching (19, 31). Likewise, glycoproteins may express heterogeneity in the occupancy of glycosylation sites (7). This diversity makes the N-glycans ideal signalling molecules, which participate in cell-cell contacts, pathogen recognition, development, signal transduction, cancer and other physiological processes (35).

2.2.1 Glycoproteins adopt their native structures in the ER

For soluble ER-synthesised proteins, folding involves minimizing the hydrophobic residue solvent exposure (36). This commonly leads to a hydrophobic protein core and a hydrophilic surface displaying the N-glycans, if they are present. Although folding is in principle instructed by amino acid sequence (37), in reality the situation is more challenging, since in the crowded cellular environment hydrophobic aggregation with

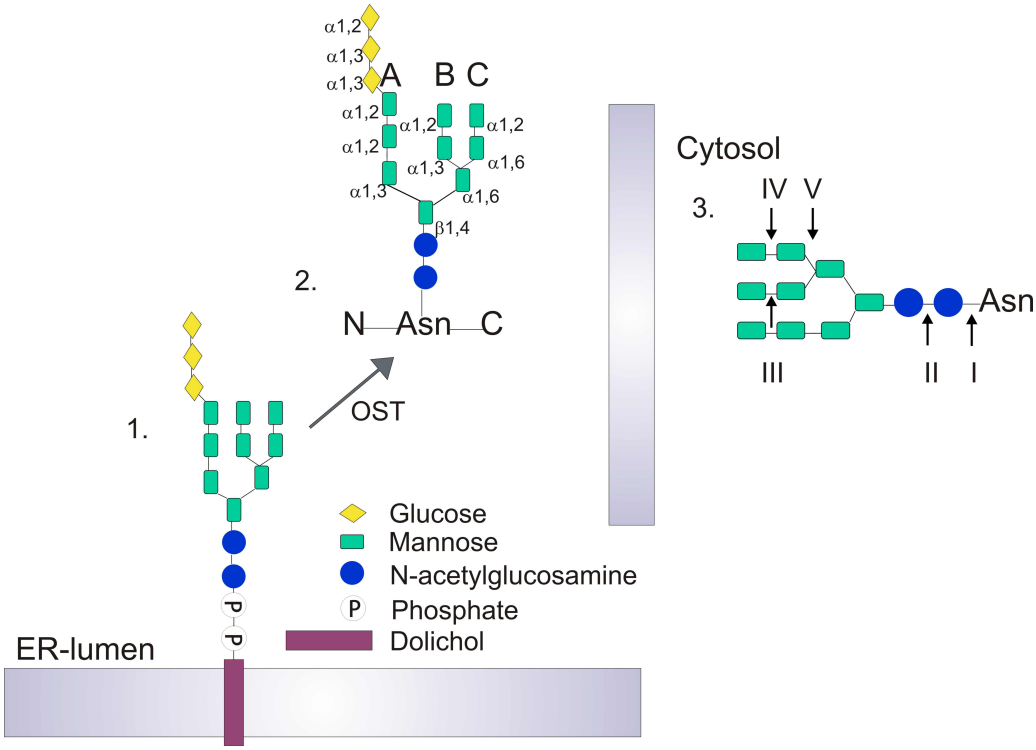

Figure 1. The initial step of N-glycosylation in the ER, structure of the N-glycan and hydrolysis in the cytosol. 1) Oligosaccharyl transferase (OST) transfers the precursor oligosaccharide from dolichol to a protein. 2) The three N-glycan branches have labels A, B and C, the N-glycan shows the conformation of glycosidic bonds. 3) The release of the free oligosaccharides in the cytosol from the ER-associated degradation targeted glycoproteins. Peptide N-glycanase (I), yields $Man_9GlcNAc_2$; Endo-β-N-acetylglucosaminidase yields $Man_9GlcNAc$ (II). The figure also describes selected reaction products of neutral α-mannosidase: $Man_8GlcNAc$ isoforms M8B (III) and M8C (IV) and $Man_5GlcNAc$, M5B (V).

other unfolded proteins is possible. Protein folding in the ER is therefore assisted by several chaperones and various folding assisting molecules, such as protein disulfide isomerase (PDI), which catalyses disulfide bond formation (38). Folding into the correct native structure is essential for proper protein function.

2.2.2 Biosynthesis of the dolichol-linked precursor involves multiple enzymes

The precursor oligosaccharide is assembled on an ER membrane lipid molecule, dolichol, through a biosynthesis pathway, which initiates in the cytosol and completes in the ER lumen. The assembly for the first seven saccharides takes places on the cytosolic side of the membrane, where glycosyl transferases add a GlcNAc residue from uridine diphosphate N-acetyl glucosamine (UDP-GlcNAc), and mannose residues from guanosine diphosphate mannose (GDP-Man) onto GlcNAc-dolichol. The biosynthesis continues on the luminal side of the ER membrane after $Man_5GlcNAc_2$-dolichol translocation. Seven glycosyl transferases sequentially extend the precursor using the

dolichol-linked mannose and glucose substrates, finally resulting in $Glc_3Man_9GlcNAc_2$-dolichol. (7, 39, 40). The mature oligosaccharide is transferred onto peptides by OST, which is a multimeric enzyme complex located nearby the translocon and the ribosome (5, 41).

2.2.3 Processing of the N-glycan glucose residues is associated with glycoprotein folding

The modifying of $Glc_3Man_9GlcNAc_2$ starts already during protein translation when glucosidase I and glucosidase II remove two glucose residues (Figure 1), (6, 8). This trimming is important for folding, since the proteins bearing monoglucosylated glycans are able to bind to membrane-bound lectin, calreticulin, or its soluble homolog, calnexin (42). Both lectins assist in folding and recruit a PDI family protein (43).

Glucosidase II also trims the final glucose, after which a protein releases it from calnexin or calreticulin (8, 42). If the protein is correctly folded, a single binding on these lectins is enough. Nevertheless, if the protein remains unfolded after release from calnexin or calreticulin, uridine diphosphate glucose:glycoprotein glucosyltransferase (UGGT) recognizes the unfolded structures (44, 45), and has strong affinity towards the core pentasaccharide $Man_3GlcNAc_2$ (46). UGGT adds a glucose residue to the glycan $Man_9GlcNAc_2$, and the protein is rebound by calnexin or calreticulin until glucosidase II removes the glucose residue again. This process of re- and deglucosylation of proteins, which is associated with rebinding to and dissociation from lectins, is entitled the calnexin/calreticulin cycle.

2.2.4 N-glycosylation has an essential role in the sorting of lysosomal proteins

In the Golgi, GlcNAc-phosphotransferase adds a GlcNAc-phosphate tag to specific glycans on proteins that are destined to lysosomes (32). During this stage of the N-glycosylation the tagged glycans are of the high mannose-type and remain this way because the tag prevents further modifications in the Golgi. The phosphorylated N-glycans are recognised in the Golgi by mannose-6-phosphate receptors (47). These receptors release their cargo in the acidic pH of endosomes, the vesicles preceding lysosomes, and recycle back to the Golgi (32). The recognition signal for the phosphotransferase action is not known: specific amino acid sequences for lysosomal proteins have not been characterised, and possibly the recognition signal is created on the protein surface during protein folding. Site-directed mutagenesis studies on lysosomal hydrolase surface residues suggest that two lysine residues situated at an appropriate distance from each other could serve as the signal (48, 49). Lysosomal proteins are also secreted extracellularly, which is common in overexpression (22), and probably due to saturation of the lysosomal transport machinery. These secreted molecules can be taken up into cells and further into lysosomes by the mannose-6-phosphate receptors on the cell membrane (47).

2.3 Unsuccessfully folded proteins are targets for ER-associated degradation

For the secretory pathway proteins, folding and its quality control are mainly limited to the ER. The quality control supervises the integrity of the folding and makes sure that the defectively folded proteins are not delivered further in the secretory pathway. In addition, the quality control sends the misfolded proteins for degradation in the cytosolic ubiquitin-proteasome system through ERAD. (9, 50).

If the folding of a protein is terminally unsuccessful, neither reglucosylation nor rebinding to calnexin or calreticulin continues and the misfolded protein is destined for degradation. ERAD recognises the misfolded substrates and translocates them into the cytosol where they are ubiquitinated by ubiquitin ligases and the peptide moiety is degraded by proteasomes (38). It is crucial for the ERAD substrates to exit the calnexin/calreticulin cycle, to be recognised by the ERAD machinery and to be rejected by the transport machinery to the Golgi. On the other hand, the ERAD machinery needs to distinguish the terminally misfolded substrates from the successfully folded ER and secretory pathway proteins. In the glycoprotein ERAD, trimming of the N-glycans has a signalling function in directing the substrates through the process (6, 8, 9, 34). The N-glycan residues important for ERAD signalling, the enzymes involved in N-glycan processing, and the molecules recognising the processed N-glycan, have been widely studied during the last decade.

2.3.1 Family 47 glycoside hydrolases trim the mannose residues on ERAD substrates

The N-glycans of the substrates of ERAD are subjected to extensive mannose trimming before they arrive in the cytosol (51, 52). In mammalian cells, ERAD substrates have their α1,2-linked mannose residues removed, which results in $Man_{5-6}GlcNAc_2$ (Figure 1; (51, 53)). Removal or exposure of the specific mannose residues may be important for sending misfolded proteins into ERAD. Removal of the terminal mannose residue from the A-branch of the glycan (Figure 1), the glucose-acceptor, permanently prevents misfolded proteins from entering the calnexin/calreticulin-cycle (54, 55). Exposure of the α1,6-linked mannose on the C-branch (Figure 1) after cleavage of the terminal α1,2-man is necessary for targeting the glycoproteins into ERAD (56).

The candidate enzymes for generating the demannosylated ERAD substrates are glycoside hydrolase family 47 (GH47) α-mannosidases of the secretory pathway (57). These include three Golgi enzymes and ER mannosidase I (ERmanI), and ER degradation enhancing mannosidase -like proteins (EDEMs) in the ER (9, 34, 57). ERmanI trims the terminal mannose in the middle branch leading to an M8B structure (Figure 1) (9), which is present on the successfully folded proteins. This α-mannosidase is also involved in ERAD, and it has been shown that inhibition of ERmanI or the disruption of its gene stabilises misfolded glycoproteins (58-60), whereas overexpression of ERmanI accelerates N-glycan trimming and degradation (61). When present at a low concentration, ERmanI results only in M8B (Figure 1), but at a high concentration the enzyme is able to remove all of the α1,2-mannoses *in vitro* (62). ERmanI has been shown

to concentrate in a special ER-derived quality control compartment, which is associated with ERAD (53). This compartmentalisation could provide a high concentration of ERmanI in the vicinity of ERAD substrates.

Other possible enzymes for modifying the N-glycans so that they would serve as signals for ERAD are EDEM1, EDEM2 and EDEM3 (8). These proteins are upregulated in ERAD and their overexpression enhances the degradation of misfolded glycoproteins (63-65). Overexpression of EDEMs also leads to mannose removal from the A-branch of the glycan (Figure 1) (55) and EDEMs are needed for the exposure of the C-branch α1,6-linked mannose (Figure 1) (56). These *in vivo* findings suggest that EDEMs have α-mannosidase activity although it remains uncharacterised *in vitro*.

2.3.2 An N-glycan signal directs glycoproteins into ERAD

The trimming of mannose residues has several effects, which direct the misfolded proteins to degradation (56, 66). The misfolded proteins need to terminally exit the calnexin/calreticulin-cycle, and extensive demannosylation is associated with this type of exit (67), while M8B (Figure 1) retains the protein in the cycle (68). Mannose trimming renders the protein a weaker ligand for calnexin and calreticulin (69), a better substrate for glucosidase II (68) and a suboptimal substrate for UGGT (70). Acceleration of protein degradation that associates with EDEM expression could be partly explained by an interaction with EDEMs and calnexin, which leads to protein exit from the calnexin cycle to ERAD (71, 72).

Extensively demannosylated glycoproteins are not recognised by the Golgi transporters VIP36, VIPL and ERGIC53 (73). The demannosylated ERAD substrates, however, have high affinity towards ER-lectins OS-9 and XTP3-B, which target the substrates for ERAD, and the availability of the C-branch α1,6-linked mannose in the terminal position (Figure1) is essential for OS-9-recognition (74).

2.4 Free oligosaccharide metabolism

The cytosol contains soluble oligosaccharides, which are not attached to proteins or lipids and thus are referred to as free oligosaccharides. The free OS pool mainly contains high mannose-type oligosaccharides, which have only mannose and GlcNAc residues, but also their phosphorylated and glucosylated derivatives are present (75-77).

2.4.1 Peptide:N-glycanase detaches N-glycans from ERAD substrates

ERAD contributes to the formation of the free OS pool. The proteasome degrades the peptide moieties of the ERAD substrates, whereas the N-glycans are separately catabolised in the cytosol and eventually in lysosomes. The deglycosylation of the ERAD targeted glycoproteins is a crucial step, since proteasomal degradation is inhibited even by a single N-linked GlcNAc (78). The deglycosylating enzyme, peptide:N-glycanase (PNGase), expresses ubiquitously in the cytosol (79). Silencing of PNGase expression decreases degradation of the ERAD substrates carboxypeptidase Y mutant and ricin toxin A in *Saccharomyces cerevisiae* (80, 81), whereas the mammalian PNGase has been

associated with the degradation of ERAD substrates in RNA interference experiments (82, 83) and PNGase inhibitor studies (84). These studies show that PNGase is involved in the degradation of glycosylated ERAD substrates, which is further supported by the finding that PNGase interacts with the proteasome through proteasome associated proteins (85, 86).

2.4.2 Free OS hydrolysis occurs in the cytosol and lysosomes

When PNGase releases the free OS from glycoproteins, it produces oligosaccharides with $GlcNAc_2$ at the reducing end. Another ubiquitously expressed cytosolic enzyme, endo-β-N-acetylglucosaminidase (ENGase), is capable of removing the terminal GlcNAc residue from the free OS (83, 87). In *Caenorhabditis elegans*, a mutation in the ENGase encoding gene led to the formation of free OS with $GlcNAc_2$ instead of $GlcNAc_1$ (88). Although ENGase trims free OS (89), this enzyme cleaves the bond between GlcNAc residues also in glycoproteins (83, 90). Currently it is unknown if ENGase contributes to the deglycosylation of ERAD substrates in addition to PNGase. In yeast, PNGase is responsible for the majority of free OS (91) and an ENGase activity is missing (92). In mammalian cells, the ENGase activity is necessary for the catabolism of free OS, since neutral α-mannosidase, the proposed enzyme for mannose trimming (10, 11), prefers the free OS with a single GlcNAc at the reducing end (93-96). Both of these free OS catabolising enzymes, ENGase and NAM, are possibly needed for the efficient clearance of free OS from the cytosol. NAM trims the mannose residues of the high mannose-type glycans yielding $Man_5GlcNAc$ (97) and also the smaller species $Man_{3\text{-}4}GlcNAc$ (10). These free OS are transported to lysosomes through a putative ATP-dependent, but autophagy-independent, saturable transporter (98). The size of the oligosaccharide has an adverse effect on lysosomal transport: the smallest molecule of the free OS species $Man_3GlcNAc$, $Man_4GlcNAc$, $Man_5GlcNAc$ or $Man_9GlcNAc$ is transported the most efficiently transported (99).

Lysosomes are the key organelles for the degradation of cellular and extracellular material. They are also the final stage for free OS hydrolysis. Mannose linkages α1,2, α1,3 and α1,6 are hydrolysed by MAN2B1 (13, 21). The second lysosomal GH38 enzyme, MAN2B2, is only able to cleave the α1,6-man bond (100). The β-linkage is degraded by β-mannosidase (101), whereas GlcNAc-GlcNAc linkage is cleaved by lysosomal chitobiase (12). In addition to finishing the hydrolysis of free OS from the cytosol, lysosomes hydrolyse the N-glycans of the lysosomally degraded glycoproteins, which are released from the protein (12).

2.4.3 A subset of free OS are released in the ER lumen

In addition to the free OS generated by ERAD, the cytosol contains free OS species, which originate from the ER lumen. The ER-fraction of the cell contains free oligosaccharides with structures similar to that of the oligosaccharide moiety of the dolichol linked N-glycan precursor (102, 103). The release of free OS into the ER lumen shares similar features with N-glycosylation: both result from an enzymatic reaction in which triglucosylated glycans are the preferred substrates (75). Due to apparent similarities

between these processes, the enzyme responsible for free OS liberation into the ER lumen has been proposed to be OST, which would transfer the precursor glycan from the transmembrane carrier molecule dolichol to water instead of a peptide (75). Consistent with this theory, the release of free OS decreased when a peptide containing a glycosylation acceptor sequence was available (102). Triglucosylated free OS in the ER are deglucosylated and delivered to the cytosol through an energy-dependent transport mechanism (104, 105).

Free OS liberation from the dolichol-linked oligosaccharides is possibly associated with regulating the availability of dolichol (39, 106). After OST transfers the glycan precursor from dolichol pyrophosphate to protein or water, dolichol pyrophosphate is recycled to the cytoplasmic side of the ER membrane. This process provides building blocks for precursor synthesis. (107). The availability of dolichol phosphate is the rate-limiting factor in DLO biosynthesis, and exogenous dolichol phosphate enhances N-glycosylation (7). The recycling of dolichol pyrophosphate is important, since *de novo* dolichol synthesis slow and also because the common dolichol phosphate pool is utilised for the synthesis of dolichol-mannose, dolichol-glucose and GlcNAc-dolichol (7, 39). The precursor for dolichol-mannose is mannose-1-phosphate, which is converted from mannose and glucose through mannose-6-phosphate (M6P). An increase in M6P promotes the release of glucosylated free OS (7) by an unknown mechanism. However, the biosynthesis pathways for dolichol-mannose, dolichol-glucose and GlcNAc-dolichol are feedback regulated and the end products of these pathways cross-regulate the enzymes of the parallel pathways (7).

2.4.4 Phosphorylated free OS are present in the cytosol

The third category of free OS is phosphorylated (OS-P) (75). The structure of OS-P was first described as $Man_5GlcNAc_2$-P (108) but later the $Man_{6\text{-}9}GlcNAc_2$-P species were also isolated (109, 110) and $Man_7GlcNAc_2$-P was characterised as the major cytosolic free OS-P species in human lymphoblastoid cells (103). $Man_5GlcNAc_2$-P is cytosolic, suggesting that a pyrophosphatase activity releases it from $Man_5GlcNAc_2$-dolichol before the oligosaccharide translocates from the cytosolic side of the ER membrane to the lumenal side (111).

2.5 GH38 α-mannosidases are involved in the building and catabolism of N-glycans

2.5.1 The classification of α-mannosidases is based on their enzymatic properties, subcellular location and sequence homology

The classification and nomenclature of α-mannosidases has followed the progress of biochemical characterisation methods. In general, α-mannosidases that participate in processing in the ER and Golgi are referred to as processing α-mannosidases, whereas lysosomal α-mannosidases are catabolic. Due to results from biochemical characterisation and subcellular fractionation studies α-mannosidases were named according to their pH optimum and subcellular origin. Further classification was

based on inhibition: α-mannosidases that were inhibited by pyranosides, such as 1-deoxymannojirimycin or kifunensine, became class 1 α-mannosidases and those that were inhibited by furanose compounds, such as swainsonine, were called class 2 α-mannosidases. (2, 3). Class 1 α-mannosidases hydrolyse α1,2-bonds, do not recognise artificial aryl substrates, and their reaction mechanism inverts the configuration of the anomeric carbon, while the reaction mechanism of class 2 α-mannosidases retains the configuration (1, 3, 112). The retaining mechanism involves two catalytic side chains, the nucleophile and the general acid/base. The catalytic nucleophile for class 2 α-mannosidases is aspartic acid (113, 114), whereas the identity of the general acid/base has not been confirmed. Class 2 α-mannosidases have more diversity for their substrates compared to class 1, since they commonly hydrolyse all types of mannosidic bonds, α1,2-, α1,3- and α1,6-man and also the aryl substrate (1-3).

The most recent α-mannosidase classification is based on sequence homologies. The database for Carbohydrate Active Enzymes, http://www.cazy.org/, comprises over a hundred glycoside hydrolase families (1). Class 1 and 2 α-mannosidases belong to families GH47 and GH38, respectively. The family GH38 contains enzymes from archaea, bacteria and eukaryotes and the biochemically characterised enzymes have α-mannosidase activity. The GH38 α-mannosidases are exo-enzymes, which cleave the terminal mannose residues from the non-reducing end of the oligosaccharide. Crystal structures have been solved for two bacterial α-mannosidases: *Streptococus pyogenes* (115), (Protein Data Bank identification code (PDB ID): 2WYH) and *Enterococcus faecalis* (PDB ID: 3LVT), and two eukaryotic α-mannosidases: *Bos taurus* lysosomal α-mannosidase (24), (PDB ID: 1O7D), and *Drosophila melanogaster* Golgi α-mannosidase (116), (PDB ID: 1HTY). In common for all GH38 crystal structures is an $(\alpha/\beta)_7$ barrel, which contains a metal ion: calcium in the *S. pyogenes* enzyme, and zinc in the others.

2.5.2 Mammalians have five ubiquitously expressing GH38 α-mannosidases

Mammalian cells have five GH38 α-mannosidases in distinct cellular compartments. Golgi II and the homologous Golgi IIx α-mannosidases (GIIAM and GIIxAM, respectively) in the Golgi (117); lysosomes contain a broad-specificity lysosomal α-mannosidase MAN2B1 and a narrow-specificity enzyme MAN2B2 (13, 100); whereas the cytosol has a neutral α-mannosidase (NAM, MAN2C1) (118, 119).

GIIAM is ubiquitously expressed in different human tissues and animals (120). This Golgi enzyme removes an α1,3- and α1,6-linked mannose residues from $GlcNAcMan_5GlcNAc_2$ yielding $GlcNAcMan_3GlcNAc_2$ (121), which is a precursor for complex-type N-glycan structures. The GIIAM activity is dependent on GlcNAc-transferase (120), which adds a GlcNAc residue to the N-glycan A-branch (122). Inhibition of GIIAM by swainsonine has been tested as a treatment for breast cancer (123, 124). As a side effect of this therapy, swainsonine also inhibits other GH38 family enzymes, which may lead to a situation, where degradation products accumulate in lysosomes due to an inhibitory effect on lysosomal α-mannosidases (120).

Another Golgi enzyme, GIIxAM has been shown to hydrolyse $Man_6GlcNAc_2$ to $Man_4GlcNAc_2$ independently of GlcNAc-transferase (125). Knocking down both GIIxAM and GIIAM is necessary for abolishing the complex N-glycan synthesis (126). Although the physiological role of GIIxAM is not well understood, this enzyme has been linked to spermatogenesis (127).

The lysosomal hydrolysis of the mannosidic linkages of the N-glycans or free OS involves two α-mannosidases and a β-mannosidase (12). The narrow specificity α-mannosidase in lysosomes, M2B2, is ubiquitously expressed in human tissues and animal species (100), although the enzyme was first referred to as the epididymal α-mannosidase according to its origin (128). The only cleavage reaction that M2B2 catalyses is the hydrolysis of the α1,6-mannose linkage (Figure 1), which is dependent on the removal of the non-reducing end $GlcNAc_2$ by the preceding actions of lysosomal chitobiase and N-acetylglucosaminehydrolase (100). This is in contrast to MAN2B1, which is capable of cleaving all α-mannosidic linkages independent of the action of hydrolases that act on GlcNAc-linkages (129).

2.5.3 Neutral α-mannosidase

In early studies the soluble fraction of a rat liver homogenate was found to contain an α-mannosidase activity, which hydrolysed the aryl α-mannopyranoside substrate, was activated by Co^{2+}, Mn^{2+} and Fe^{2+} and had a pH-optimum of 6.5 (130). The α-mannosidase with these properties was first purified from rat liver cytosol (118) and several other tissues and organisms (95, 131, 132, 133). According to its intracellular location or pH optimum it was named cytosolic or neutral α-mannosidase (2, 134).

Although the NAM enzyme activity was discovered in the cytosol, an activity with similar characteristics was identified and isolated also from the ER-fraction of rat liver in subcellular fractionation studies (14, 15). Cloning of the NAM gene was based on the amino acid sequence of the ER-fraction protein, thus the first complementary DNA (cDNA) sequence for NAM was referred to encode an ER α-mannosidase (15).

The characterisation of the NAM enzyme activity in tissues or cells and the native sources of the activities of the purified enzymes suggest that NAM digests the high mannose-type substrates and yields $Man_5GlcNAc$ (11, 97). Silencing of human NAM with small interfering RNAs (siRNA) increased the amount of $Man_9GlcNAc$ (11) and consistent with this, overexpression of the mouse NAM decreased the amount of $Man_9GlcNAc$ (96). The reducing end structure of the oligosaccharide substrate is important for NAM, since the $GlcNAc_2$ containing oligosaccharides and the peptide-bound N-glycans are poor substrates. (93, 95, 131, 133). These substrate preferences together with the cytosolic origin of the enzyme activity propose that NAM would participate in cytosolic free OS metabolism.

The cellular effects of human NAM have been studied by siRNA silencing NAM expression in cancer cell lines. One of the effects of silencing was that cancer cells became less malignant; they were less tumorigenic and less metastatic (17). Specifically, the silencing correlated with enhanced E-cadherin expression (17). E-cadherin mediates

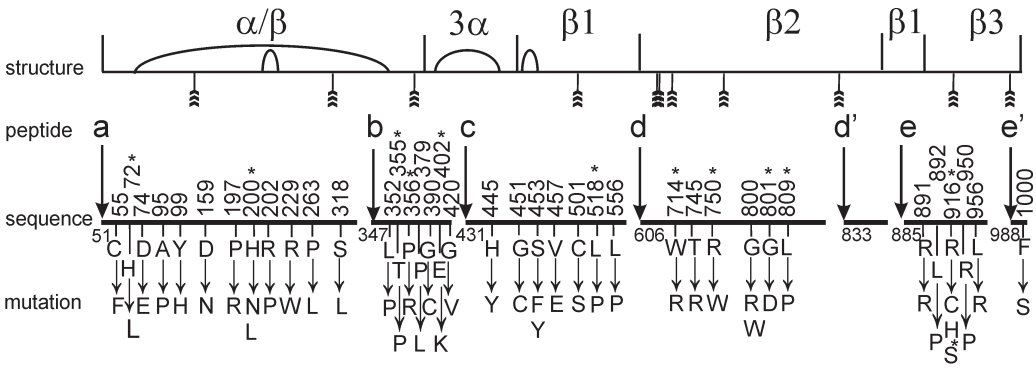

Figure 2. Schematic depiction of the human MAN2B1 sequence, missense mutations and structure. Disulfide bridges (curves) and predicted N-glycosylation sites (arrows) are marked on the structure. The proteolytic processing sites are modelled from the bovine MAN2B1 crystal structure (24). *The mutations, which have been previously characterised (22, 29, 30). The figure is reprinted from the original publication III.

adhesion between epithelial cells (135), and the decreased expression of E-cadherin is associated with tumours (136). NAM silencing also led to the loss of lamellipodia (17), which are cell surface protrusions important for cell spreading and motility (137). NAM silencing also had an effect on telomere shortening (138), which is an essential process for regulating the life span of cells (139). When NAM expression was suppressed in esophageal cancer cells, the cells arrested in mitosis, which was accompanied by microtubule disarrangement and apoptosis (140).

2.5.4 Lysosomal α-mannosidase

The human *MAN2B1* gene comprises 24 exons (141). The gene encodes for a MAN2B1 peptide of 1011 amino acids, but after cleavage of the signal peptide in the ER the length of the protein is 962 amino acids. The mature lysosomal enzyme is a dimer (23). During transport to the lysosome, MAN2B1 is proteolytically processed after the *trans*-Golgi compartment, and produces on a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) five shorter than full-length peptides a, b ,c, d and e (Figure 2), which have been N-terminally sequenced (142). Eventually, the crystal structure of the bovine enzyme suggested two additional proteolytic sites in the d- and e-peptides (24). When overexpressed, MAN2B1 is extracellularly secreted as the full-length enzyme, which is active (22).

The crystal structure of bovine MAN2B1 revealed that the enzyme consists of four domains: an N-terminal α/β-domain, and three all-β-domains. The N-terminal α/β-domain, which is formed by the a- and b-peptides, contains the active site. (24). The catalytic nucleophile for bovine MAN2B1was probed for with a covalently bonded substrate analogue (114), and the found nucleophile corresponds to residue D196 in the human enzyme. The bovine MAN2B1 crystal structure contains a zinc ion in the active site, where it is coordinated by two histidine (H72, H446) and aspartic acid

residues (D74, D196). The active site also has a Tris molecule, which most likely mimics substrate binding. (24).

During its synthesis in the ER, MAN2B1 is N-glycosylated (22). Bovine MAN2B1 has eight N-glycosylation sites (13), which contain all types of N-glycans: high mannose-, complex- and hybrid-types (143). Although N-glycans are usually difficult to see in crystal structures due to their relatively high mobility, six of these sites show at least the first GlcNAc residue in the crystal structure. Of these, the N-glycan at N497 is exceptionally well-ordered, showing two GlcNAc and seven mannose residues. (24). The high resolution of the N497 glycan suggests that it is structurally important. This MAN2B1 N-glycan site is also highly conserved among eukaryotes (143).

The lysosomal environment is different from the ER and the Golgi, where the lysosomal proteins are synthesised. Lysosomal enzymes must maintain their functionality at acidic pH and in the presence of proteases and glucoside hydrolases. Since the activity of the nascent, ER-synthesised lysosomal hydrolases would be damaging for the cells, inactivation mechanisms during transport must exist. At a neutral pH similar to the ER, MAN2B1 has only residual activity, but the enzyme is exceptionally stable and survives heating at 60 °C for 20 minutes (13). However, the pH optimum for MAN2B1 is below pH 5 (144). The bovine MAN2B1 crystal structure suggests that the low-pH activation of MAN2B1 would occur via the relaxation of three separate ion-bonding networks (24). Proteolytic processing is not associated with MAN2B1 activity, since the extracellular full-length enzyme is active (21, 22).

2.6 α-mannosidosis and lysosomal storage disorders

2.6.1 Lysosomal storage disorders

The lysosomal degradation of proteins, carbohydrates and lipids involves over 60 hydrolases. These lysosomal hydrolases, or occasionally also lysosomal transporters or trafficking mechanisms, are affected in lysosomal storage disorders (LSDs), a group of diseases comprising more than 50 diseases. (145). Due to a missing enzyme activity, metabolic products accumulate into lysosomes leading to enlarged vacuoles, which are characteristic of LSDs. The biochemical mechanisms by which the accumulation leads to pathology are under extensive study. Recent findings suggest that the accumulation of metabolic products alters various signalling pathways, affects calcium homeostasis, lipid biosynthesis, the trafficking of lipids and their receptors, impairs autophagy and causes inflammation (25, 146). Genetic defects in LSDs and the enzymes involved are reasonably well-characterised (26, 129).

LSD-causing mutations are mostly monogenic: they are on a single gene encoding a lysosomal enzyme. Mutations may completely destroy the enzyme activity, but occasionally enzymes express low activity. (147). LSDs are commonly inherited in an autosomal-recessive manner, although recessive chromosome X-linked diseases exist (26). Although individual LSDs are rare diseases, their collective incidence is estimated to be 1/5000 - 1/8000 live births (148-150). The onset of disease varies from an infantile

form, which is usually fatal, to juvenile and adult forms, which typically have milder symptoms (147). The broad range of symptoms is characteristic for LSDs, even within a single disease such as α-mannosidosis (27). Although individual patient mutations in LSDs are often well-described, genotype-phenotype correlations are challenging to show.

For most LSDs, the current therapy can only alleviate the symptoms, not cure the disease. However, a few LSDs are treated by the administration of an intact enzyme (151). This enzyme replacement therapy is available for Gaucher, Fabry and Pompe diseases and for selected mucopolysaccharidosis types (references within 151). An occasional side effect of enzyme replacement is the development of antibodies against the exogenous enzyme. Other challenges are the delivery of recombinant enzyme into skeletal muscle cells (152), and to the central nervous system through the blood brain barrier (153). Hematopoietic stem cell transplantation is the second option for introducing healthy enzyme into patients (151). So far, this therapy has only been clinically tested in small scale (references within 151). Small molecule inhibitors provide different treatment mechanisms for LSDs. In chaperone therapy, the inhibitor molecule assists in enzyme folding, and this strategy may increase the enzyme activity in lysosomes. Chaperone therapy is limited to patients with missense mutations. Substrate reduction therapy aims to reduce the formation of the accumulating substrate.

2.6.2 Mutations in the *MAN2B1* gene cause α-mannosidosis

In α-mannosidosis, the missing MAN2B1 activity results in the accumulation of unprocessed mannose containing oligosaccharide material. This rare LSD has an estimated incidence of 1/500 000 live births (27). The clinical picture of the disease is diverse, the frequent symptoms being infections, skeletal deformation, muscle weakness, mental retardation and hearing problems. (27). Clinically α-mannosidosis is divided into three subtypes according to the age of onset of the disease and the symptoms: mildly affected patients older than 10 years, quite severely affected patients younger than 10 years and the severe infantile form that leads to early death. The classification is often not straightforward, since the patients have a variety of symptoms. (27). In addition to humans, α-mannosidosis occurs in cattle (13, 154), cat (144, 155) and guinea pigs (156, 157). A mouse model for the disease resulted in the accumulation of oligosaccharides in various tissues and organs, but the clinical symptoms typical for the human disease were missing (158).

The diagnosis for α-mannosidosis is based on an enzyme activity measurement for MAN2B1 from patient cells. Suggestive diagnostic means are the analysis of unprocessed mannose glycans in the urine and a microscopic examination for swollen lysosomes. (27). Sequencing the patient's *MAN2B1* alleles provides information on the disease-associated mutations (28, 159). Since α-mannosidosis is inherited in an autosomal recessive manner, both alleles carry mutations although the mutations on the two alleles are often different (28). The mutation types that have been discovered in α-mannosidosis are insertions, deletions, missense, nonsense and splicing site mutations, duplications and inversions (27, 28, 159). Nonsense mutations introduce an

additional stop codon which leads to premature termination of translation. Missense mutations result in amino acid changes, but synthesis of the protein still occurs, whereas the other mutation types mostly lead to a situation where the protein is not synthesised at all. The currently known 124 mutations spread through the whole length of the *MAN2B1* gene (28). Over 30 missense mutations have been reported (22, 28-30, 159)}. Although most of them are family-specific, the mutation resulting in p.R750W occurs more frequently (159), in over 20% of European patient alleles (28).

Missense mutations may be defective in various mechanisms. The active site mutation resulting in p.R220H in bovine MAN2B1 impairs substrate binding and affects the orientation and stability of the reaction nucleophile (24). In human MAN2B1, the mutations resulting in p.H200L or p.H200N remove hydrogen bonding, which destabilises the active site (29, 30). The mutation resulting in p.H72L affects zinc binding and leads to loss of enzymatic activity (23, 24). The variant enzymes p.R220H and p.H72L, however, are transported to the lysosome, which indicates that their folding in the ER has been successful, although they do not have α-mannosidase activity (22). Some of the MAN2B1 missense mutations interfere with folding. The bovine mutation leading to p.F320L disturbs the dimer interface interactions, whereas the human mutation leading to p.R750W affects the interactions between the d- and e-domain interface (22, 24). Despite the available genetic data, phenotype-genotype correlations have been difficult to identify in α-mannosidosis (28). Even siblings who carry an identically mutated MAN2B1 gene may have a very different disease outcome (23, 28).

The current treatment for α-mannosidosis is mostly the non-specific management of symptoms (27). Patient trials for specific treatments for α-mannosidosis thus far only include bone marrow transplantation, which improved the condition of four patients (160). This method is most beneficial at an early age and has high risk of morbidity and mortality, and therefore needs careful risk assessment (27). Enzyme replacement therapy has been tested on a α-mannosidosis mouse model (161) and in guinea pigs (162). The treatment decreased the amount of accumulated material in both animal models (161, 162).

3. AIMS OF THE STUDY

1. The cellular role of human NAM

   a) Production of pure, homogenous NAM for biochemical and structural studies.
   b) Characterisation of NAM with potential substrates and cofactors *in vitro*.
   c) Solving the intracellular location of NAM *in vivo*.

2. The molecular pathology of α-mannosidosis

   a) Characterisation of the cell biological effects of α-mannosidosis associated missense mutations
   b) Modelling of the human MAN2B1 three dimensional structure and structural analysis of the missense mutations.
   c) Compiling the results for α-mannosidosis mutation characterisation with *MAN2B1* mutation mapping, geographical distribution of disease alleles and clinical information into a public database.

4. SUMMARY OF THE MATERIALS AND METHODS

The materials and methods are described in more detail in the original publications I-IV.

4.1 Cloning, expression and purification of human NAM (I)

The complementary DNA encoding human NAM was cloned from human fibroblast cDNA by polymerase chain reaction (PCR) with primers homologous to a GeneBank human NAM sequence (accession number: NM_006715). The PCR-product was subcloned into a pCR2.1-TOPO vector (Invitrogen) and sequenced. For *Pichia pastoris* expression, the NAM cDNA was ligated into the pPIC9 vector (Invitrogen) under a yeast secretion signal, and into the pPIC3.5 (Invitrogen) for intracellular expression. In addition to these constructs for native NAM expression, NAM with a C-terminal six-histidine tag (6xHis) and NAM with an N-terminal 6xHis followed by a tobacco etch virus protease (TEV) recognition site were inserted into pPIC3.5. The resulting constructs were integrated into the *P. pastoris* strain GS115 chromosome, under the methanol inducible alcohol oxygenase promoter. For expression in mammalian cells, the NAM cDNA was inserted into the vectors pcDNA3.1(-) (Invitrogen) or pEGFP-N1 (Clontech) in frame with Enhanced green fluorescent protein (EGFP).

Expression of the NAM constructs was tested in bottle cultures, whereas large scale expression was performed in a 5 l biocultivator. Expression in *P. pastoris* was performed according to the 'Pichia expression kit' (Invitrogen) and the 'Guidelines for Pichia fermentation' manuals (Invitrogen). In a typical fermentation, *P. pastoris*/6xHis-TEV-NAM was precultured to an optical density value of 3 (at wavelength 600 nm) in 150 ml of glycerol containing buffered yeast complex medium. Cultivation in the bioreactor was initiated by adding the preculture to 2 l of basal salts medium containing trace salts and glycerol. During fermentation, the temperature was adjusted to 29 °C, the pH was 5.5 and dissolved oxygen was above 20%. Cells were grown to a density of 110 g/l during the glycerol batch and feed phases, after which NAM expression was induced by a gradual increase of methanol feed. In a typical fermentation, the α-mannosidase activity reached a stable level after a methanol feed of 830 ml. At this stage the cell density of the culture was of 300 g/l.

Cells from the *P. pastoris*/6xHis-TEV-NAM fermentation were lysed with glass beads, and the α-mannosidase activity was precipitated from the clarified lysate in 42% ammonium sulphate. The precipitate was solubilised and the buffer was changed using a HiPrep 26/10 column (GE Healthcare). For affinity purification with a histidine tag, the desalted protein fractions were applied into a HiTrap cobalt chelating column (GE Healthcare) and the column-bound NAM was eluted with an imidazole step gradient. The histidine tag was cleaved from 6xHis-TEV-NAM by TEV digestion, and following the digestion, the sample was applied to the second cobalt chelating chromatography, in which the successfully TEV-cleaved NAM remained in the flow-through fraction. For

NAM-antiserum production, NAM from the second cobalt chelating chromatography was purified with gel filtration through a HiLoad 26/60 Superdex 200 column (GE Healthcare). The purification was monitored for the α-mannosidase activity and the size and purity of NAM were analysed on SDS-PAGE. The size of the native NAM was determined by the HiLoad 26/60 Superdex 200 column with the molecular weight markers for albumin, aldolase, catalase, ferritin and thyroglobulin (GE Healthcare).

4.2 Biochemical characterisation of NAM (I)

For α-mannosidase activity measurements, protein samples were incubated for 30 min at 37 °C in a reaction containing 100 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 6.5, 1 mM $CoCl_2$, and 2 mM p-nitrophenyl α-D-mannoside (PNP-α-man). The reaction was stopped with an equal volume of 13 mM glycine buffer at pH 10.7, 67 mM NaCl and 83 mM Na_2CO_3. The absorbance of p-nitrophenyl was measured at 405 nm. A similar protocol was applied to determine the pH optimum, except NAM was preincubated for 15 min at 37 °C min in either 20 mM acetate, MES or N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES) buffers which had their ionic strength adjusted to 20 mM with NaCl and contained 0.167 mM $CoCl_2$. For determining the effect of divalent cations, the preincubation of NAM was carried on in 167 mM MES, pH 6.5, with 0.167 mM cation ($CaCl_2$, $CoCl_2$, $CuSO_4$, $FeSO_4$, $MgCl_2$, $MnCl_2$ or $ZnCl_2$).

A qualitative analysis of hydrolysis was performed with radiolabelled $Man_9GlcNAc$ and $Man_9GlcNAc_2$. The reactions containing 1 µg of NAM, 20 000 disintegrations per minute (dpm) of oligosaccharide, 20 mM HEPES, pH 7.2, 110 mM potassium acetate, 2 mM magnesium acetate and 1.8 mM $CoCl_2$ in a reaction volume of 100 µl, were incubated for 18 h at 37 °C. A similar protocol was applied to the time-dependent analysis of hydrolysis with $Man_9GlcNAc$. For analysing the effect of the cationic metals, 0.2 µg of NAM was incubated for 15 min at 37 °C in a 10 µl reaction volume containing 100 mM MES, pH 6.5, and 0.2 mM cation ($CaCl_2$, $CoCl_2$, $CuSO_4$, $FeSO_4$, $MgCl_2$, $MnCl_2$ or $ZnCl_2$) or with 50 mM ethylenediaminetetraacetic acid (EDTA). An equal volume of substrate mixture was added (20 000 dpm of $Man_9GlcNAc$ in 100 mM MES, pH 6.5) and the incubation was continued for 25 min. The reactions were stopped by heating for 5 min at 100 °C, evaporated until dry, dissolved in acetonitrile/water 70:30 (V/V) and analysed by high pressure liquid chromatography on an Asahipak NH2P-50 column (Asahi), with a solvent gradient of acetonitrile/water 70:30 (V/V) to 50:50 (V/V). Oligosaccharides were detected by their radioactivity and identified by comparing their retention times with oligosaccharide standards (163).

4.3 Subcellular localisation studies of NAM and MAN2B1 variants (I, III, IV)

Chinese hamster ovary cells (CHO-K1), African green monkey kidney cells (COS-7) and human cervical carcinoma cells (HeLa) were grown according to standard cell culturing procedures in Dulbecco's modified Eagle medium supplemented with 1% glutamine

or GlutaMAX (GIBCO), 10% fetal bovine serum, penicillin/streptomycin and 1% non-essential amino acids (for CHO-K1) at 37 °C in 5% CO_2.

The plasmid DNA was transfected in the cells growing on glass cover slips with Cellfectin (Invitrogen) or JetPEI™ (Polyplus-transfection) reagents, according to the manufacturer's instructions. After 24-48 hours of transfection, protein synthesis was arrested with 50 µg/ml of cycloheximide, and the cells were fixed with 4% paraformaldehyde, quenched with 50 mM NH_4Cl and permeabilised with 0.1% Triton-X 100. Unspecific binding was blocked with 0.5% bovine serum albumin; the cells were labelled with primary antibodies, and further stained with Alexa-labelled secondary antibodies (Invitrogen). The samples were mounted in polyvinyl alcohol or in ProLong® Gold (Invitrogen). For staining lysosomes, cells were fixed with 100% methanol and blocked with 0.5% bovine serum albumin, in a solution containing 0.2% saponin. After this, the staining protocol was similar to paraformaldehyde fixed cells, except that all solutions contained 0.2% saponin.

The fixed cells were imaged with the confocal microscopes Leica TCS SP or SP2 AOBS. The optically sectioned images of the transfected cells were captured using the appropriate lasers and settings for the emission and absorbance for the Alexa stains. Colocalisation of the target protein and the organelle marker protein was statistically analysed by selecting the area for a single transfected cell in all optical sections, and calculating the Pearson correlation coefficient value and its statistical significance with the Costes algorithm (164), which allows discrimination of true pixel overlapping from random ones. This algorithm is provided by the Colocalisation test plugin in the ImageJ image processing program (165). A detailed description for each immunofluorescence experiment is presented in Table 1.

4.4 Colocalisation of NAM with proteasomes

A stable cell line for NAM, HeLa-NAM, was grown to 50-60% confluency on glass cover plates. The cells were transfected using the JetPEI™ reagent (Polyplus-transfection) with the plasmid DNA containing a proteasome subunit LMP2 fused with Green fluorescent protein (LMP2-GFP) (166), or the fragment of a Golgi protein, galactosyltransferase, fused with EGFP (GT-EGFP) (167). The plasmid encoding GT-EGFP was a gift from Dr. Sakari Kellokumpu (the plasmid was originally derived from Dr. R. Tsien, San Diego, CA) and the plasmid for LMP2-GFP was a gift from Dr. Jacques Neefjes. Immunofluorescence staining began 24-30 h after transfection. The cells were treated with a paraformaldehyde-fix protocol as described in 4.3. Unspecific binding was blocked in 1% bovine serum albumin for 1 h and the cells were stained with a rabbit antiserum against human NAM for 1 h. The secondary staining was performed for 1 h with 1:500 diluted goat anti-rabbit Alexa 633 antibody, and ProLong® Gold was used for mounting. Optically sectioned images were captured with a Leica TCS SP5 confocal microscope using an HCX APO CS 63.0x/1.3 Glyc objective, and the settings 488 nm and 633 nm for excitation and 497-568 nm and 642- 750 nm for emission. A statistical analysis of colocalisation was performed on Z-stack images for ten cells originating from three separate immunostaining experiments by ImageJ (165). Thresholds for

the images were set automatically and the statistical significance of colocalisation was calculated by a randomisation test with 100 iterations (164).

4.5 Structural analysis of human MAN2B1 (III, IV)

We constructed a model of the human MAN2B1 protein structure by aligning its peptide sequence (AAH00736.1) with the bovine MAN2B1 (Q29451) and using the bovine crystal structure (PDB ID:1O7D) (24) as template. Sequence alignments and the initial modelling were performed with the Bodil modelling environment (168). Comparative modelling with Modeler 9v7 (169) was used to calculate five models for human MAN2B1, and the model with the lowest objective function was validated with Procheck (170). This model was used as a template to analyse the structural effects of the α-mannosidosis mutations. The mutations were manually modelled in Coot (171), using the known conformational libraries for amino acid residues, and by choosing the conformation most similar to the wild type enzyme.

4.6 α-Mannosidosis database (IV)

The thesis describes the α-mannosidosis database, which will be available at http://amamutdb.no. The database has been generated as part of two EU-consortium projects, which aimed to increase our knowledge on the pathophysiology of α-mannosidosis and to develop new therapies for the disease. The database compiles background data for the disease, genetics and MAN2B1 protein, contains a constantly updated list of published α-mannosidosis mutations, the results for experimental characterisation of MAN2B1 variants and clinical description of patients. The database is designed to be both interactive and contextual; it allows searching with several filtering options, which retrieve data also from the parallel sections of the database. Currently the database is password protected and thus accessible for the consortium members only. To protect the anonymity of the patients, the password-access policy will continue to apply to the patient and clinical data when the database is in publically available. For the same reason, patients are represented in the database only by confidential code numbers.

5. SUMMARY OF THE RESULTS

5.1 Characterisation of human neutral α-mannosidase (I, II)

5.1.1 Cloning of the human NAM cDNA, expression in *Pichia pastoris* and purification of the recombinant enzyme

To produce human NAM in large-scale, we cloned the cDNA for human NAM and expressed the enzyme in *P. pastoris* with an N-terminal histidine tag. The position of the tag had a strong effect on NAM expression: the clones with the N-terminal histidine tag had 20 times more α–mannosidase activity than the untagged or C-terminally histidine tagged NAM clones (data not shown). The vector pPIC3.5 lacks the yeast secretion signal and NAM expressed intracellularly in *P. pastoris*, whereas an expression trial with a yeast secretion signal did not produce α-mannosidase activity into the medium. The size of NAM on SDS-PAGE was of the predicted length (I, Figure 2B), suggesting that NAM was not glycosylated although it has four N-glycosylation sites. The signal prediction methods SignalP (172) and WoLF PSORT (173) did not predict any ER targeting signal sequences in NAM.

Purification of NAM involved ammonium sulfate precipitation from a *P. pastoris* cell lysate, cobalt chelating chromatography to enrich the histidine tagged enzyme, removal of the purification tag by TEV digestion and separation of the untagged NAM from 6xHis-TEV-NAM by a second round of affinity chromatography. The NAM recovered from the purification was pure and homogenous, and its molecular size on SDS-PAGE corresponded well with the predicted size of 112 kilodaltons (kDa) (I, Figure 2B). The yield from the purification was typically 3-8 mg/l of fermentation. To study the native state of NAM, we applied the purified enzyme onto size exclusion chromatography column together with protein standards (I, Figure 2A, page). The NAM retention time (151.9 min) suggested that NAM has a tetrameric quaternary structure.

5.1.2 NAM hydrolyses the $Man_9GlcNAc$ high mannose glycan

NAM has been proposed to trim the cytosolic free OS after PNGase and ENGase action (10, 77). Therefore we studied the time-dependent hydrolysis of the putative natural substrate $Man_9GlcNAc$ with the purified enzyme preparation of NAM. Analysis of the reaction products showed that NAM trimmed $Man_9GlcNAc$ to $Man_5GlcNAc$, and the reaction proceeded through the intermediates $Man_{6\text{-}8}GlcNAc$ (I, Figure 3). After 18 h of reaction time, the main products were free mannose and $Man_5GlcNAc$, but lesser amounts of $Man_6GlcNAc$ and $Man_4GlcNAc$ (I, Figure 4A) were also present. In addition, we studied the hydrolysis of the nine-mannose substrate with two GlcNAc residues at the reducing end. Even after the extended reaction time, the most abundant reaction product was $Man_8GlcNAc_2$, and only small amounts of $Man_{6\text{-}7}GlcNAc_2$ were present (I, Figure 4B).

We studied the effects of different cations and EDTA on the NAM reaction with $Man_9GlcNAc$ and PNP α-man. With the cations Fe^{2+}, Co^{2+} and Mn^{2+}, the NAM reaction yielded the main product $Man_5GlcNAc$ via $Man_{6\text{-}8}GlcNAc$. The cations Mg^{2+}, Ca^{2+} and Zn^{2+} had a similar effect as did EDTA or preincubation without metals, and NAM trimmed a single mannose residue yielding the $Man_8GlcNAc$ isomer M8C (Figure 1). The only metal, which completely inhibited the hydrolysis of $Man_9GlcNAc$, was Cu^{2+}. Based on this hydrolysis analysis we classified the metals into three groups. Activating metals produced more $Man_5GlcNAc$ than M8C (Fe^{2+}, Co^{2+}, Mn^{2+}), null effect metals more M8C than $Man_5GlcNAc$ (Mg^{2+}, Ca^{2+}, Zn^{2+}) and an inhibiting metal led to a total block of hydrolysis (Cu^{2+}) (I, Table I). This grouping was applicable also to PNP α-man substrate studies, where we could classify the metals by the relative rate of hydrolysis. For PNP α-man, the relative hydrolysis rate was 23% higher with Fe^{2+}, than with Co^{2+}, and also Zn^{2+} had an activating effect (I, Table II).

We determined the pH profile for NAM with PNP α-man in the pH range of 4.25 – 8.0. The optimal pH for human NAM in this reaction was between 6.25 and 6.75 (I, Figure 5), which is in good agreement with values for the hen and bovine NAMs, 6.5 and 6.7, respectively (95, 133)

5.1.3 NAM is a cytosolic enzyme with a tendency to colocalise with proteasomes

NAM has been isolated both from the cytosol and the ER by density gradient fractionation (14, 94, 118, 119). To solve the intracellular localisation of this α-mannosidase, NAM expressing immunostained cells were imaged with a confocal microscope. Staining for NAM spread through the cytosol but did not colocalise with the ER (I, Figure 6B) that was labelled with an ER-retaining EGFP –KDEL. The Costes analysis confirmed the absence of NAM in the ER, giving the observed Pearson correlation coefficient (R_{obs}) of 0.21. Comparison of the cellular distribution for the immunostained NAM and NAM-EGFP -fusion (I, Figure 6A) showed that these two stains colocalised, and thus the antiserum for NAM was specific for this α-mannosidase.

The cytosolic appearance of both human and endogenous NAM in CHO-K1 (I, Figure 6B and C) was punctuated and thus different from the smooth staining of the cytosolic protein represented in our study by soluble EGFP (I, Figure 6D and supplementary data, Figure 2). Therefore, we studied if NAM interacted with membrane structures other than the ER. Since the NAM reaction product, $Man_5GlcNAc$, is transported into lysosomes, NAM could be related both to those organelles and autophagosomes, which are cytosol-to-lysosome targeting vesicles (174). We detected these organelles with a lysosomal and late endosomal marker EFGP-Rab7 (175) and an autophagosomal marker LC3 that was fused with Cyan fluorescent protein (176), and costained the cells for NAM. However, the images demonstrated that NAM was absent both from lysosomes and autophagosomes (I, Figure 6E and F).

Due to the possible role for NAM in the hydrolysis of N-glycan moieties released from the ERAD substrates that are proteasomally degraded, proteasomes are possible candidates for NAM interaction partners. Therefore, to further investigate the putative interactions that would result in the granular appearance of NAM in the immunofluorescence

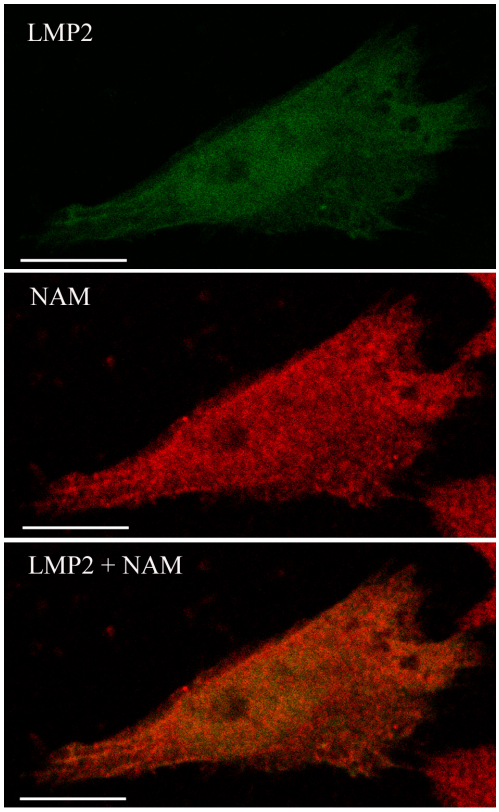

Figure 3. Intracellular localisation of NAM in HeLa-NAM cells. Immunofluorescence labelling of NAM (red), proteasomal marker LMP2 (green) and overlay of these two images. Scale bar corresponds to 10 µl.

studies, we studied the colocalisation of NAM with proteasomes. In addition to CHO-K1 cells that transiently expressed human NAM, we immunostained NAM and visualised proteasomes by expressing LMP2-GFP in the HeLa-NAM cell line. The overlay images for immunostained NAM and proteasomes in HeLa-NAM show colocalisation (Figure 3) and that the two stains produce similar patterns in the cytosol. The Costes test for ten cells confirmed the colocalisation: the mean value for R_{obs} was 0.76, while 0.40 would have indicated random colocalisation (R_{rand}). The results were similar in CHO-K1 cells: the mean value for seven cells gave a Pearson correlation coefficient of 0.74 for NAM and proteasomes, while 0.44 would have indicated random colocalisation. The non-overlapping control is shown in HeLa-NAM cells, which expressed the Golgi marker GT-EGFP. The NAM stain covers the cytosol, while GT-EGFP visualises the Golgi stacks (Figure 4). The overlay image shows distinct localisation of NAM and GT-EGFP.

5.1.4 NAM overexpression associates with protein underglycosylation and enhanced ERAD

The cellular effects of NAM were studied *in vivo* in the HeLa-NAM cells, which had a 72-fold increase in NAM expression compared to the wild type HeLa cells. Evaluation of free oligosaccharides revealed that their total amount had increased in HeLa-NAM and also the composition of free OS had changed: while the wild type HeLa cells had $Man_{5\text{-}9}GlcNAc$, HeLa-NAM had also $Man_{2\text{-}4}GlcNAc$ (II, Figure 3, Table I). In addition to

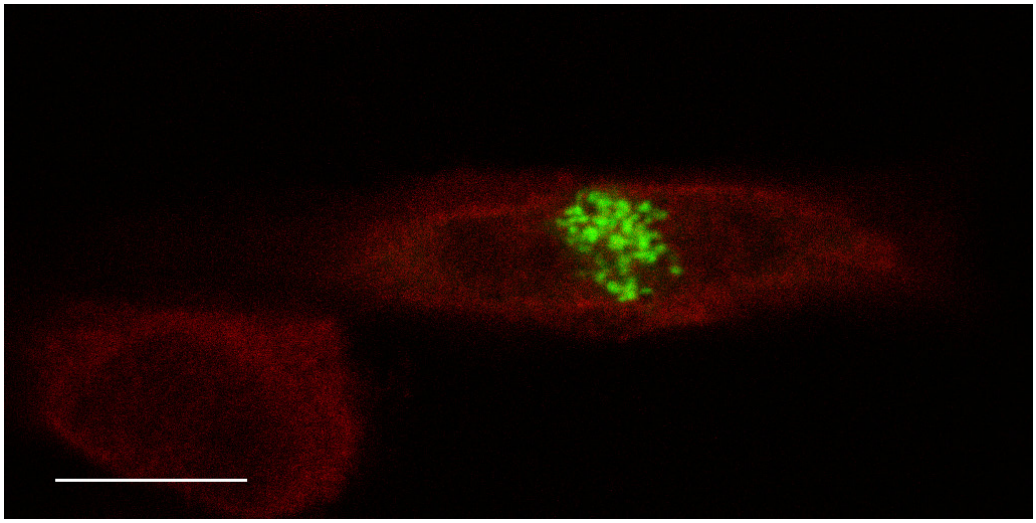

Figure 4. Immunofluorescence image of NAM and the Golgi in HeLa-NAM cells. Overlay of images shows NAM (red) and the Golgi galactosyltransferase fusion with EGFP (green). Scale bar corresponds to 10 μm.

these changes in the cytosolic free OS pool, the overexpression of NAM also correlated with an increase in anti-ubiquitin binding proteins and the accumulation of the ERAD substrate Null Hong Kong variant of α-antitrypsin (NHK) (177) (III, Figure 4). Inhibition of proteasomes increased the amount of anti-ubiquitin binding proteins and NHK both in wild type HeLa and HeLa-NAM, however the increase was more extensive in HeLa-NAM, which suggests that NAM overexpression increases the amount of the ERAD substrates. Analysis of the N-glycans on NHK expressed in HeLa-NAM revealed that this protein was underglycosylated when compared to NHK in the wild type cells (II, Figure 5). Since the same applied to β1-integrin (II, Figure 5), a cell membrane protein passing through the secretory pathway, the underglycosylation did not occur in the Golgi but earlier in the secretory pathway.

Further experiments on the protein underglycosylation in HeLa-NAM showed that the protein linked N-glycans carried a single glucose residue, and that the dolichol-linked N-glycan precursors were lacking all three glucose residues (II, Figure 6). Since inhibition of ER glucosidases did not alter the glycosylation, the underglucosylation must have derived already from the dolichol-linked oligosaccharide, and the single glucose in the N-glycans resulted from the glucosyltransferase action during the calnexin/calreticulin cycle. Finally, the analysis of the dolichol-linked oligosaccharide building blocks showed that the amount of M6P increased while the amount of UDP-Glc decreased during NAM overexpression (II, Figure 7).

5.2 Molecular and cell biological effects of α-mannosidosis associated MAN2B1 mutations (III, IV)

Recently, the gene level characterisation of 83 patient mutations and the analysis of the α–mannosidase activity of heterologously expressed missense α–mannosidosis variants were accomplished (28). The novel missense mutations are particularly interesting, because predicting their pathogenic effect is difficult. The biochemical or cell biological characterisation has been reported for 12 missense mutations (22, 29, 30). This thesis reports the molecular consequences of 35 α-mannosidosis associated mutations, including 29 novel missense mutations, which were identified by Riise Stensland *et al.* 2011. In addition, the thesis describes the α-mannosidosis database, which compiles the old and new results for mutation characterisation and clinical data.

5.2.1 MAN2B1 α-mannosidosis variants reside in the ER and lysosomes

To study the molecular pathology of the α–mannosidosis associated mutations, we investigated by immunofluorescence microscopy, which mutations allow lysosomal targeting of MAN2B1 and which arrest the enzyme in the ER. For the analysis, each variant construct was transiently expressed in HeLa cells and the fixed cells were immunostained with antibodies against MAN2B1 and lysosome-associated membrane protein 1 (LAMP1). The lysosomal localisation was easy to see from the yellow vacuole staining in the overlay images of MAN2B1 and LAMP1 (Figure 5; III, Figure 3). Colocalisation was confirmed by the Costes method for seven cells of each variant. The mean values for R_{obs} were between 0.63 and 0.81 for MAN2B1 variants, while the mean values for R_{rand} were between 0.13 and 0.20 (III, Supplementary material, Table S1). The respective mean R_{obs} value for wild type MAN2B1 was 0.75, whereas the mean R_{obs} for the known ER retaining variant, R750W was 0.32, and thus close to the random value. The variants, which were missing from lysosomes, were costained for the ER protein PDI and analysed similarly as MAN2B1 stained variants. Mean R_{obs} values for ER variants were from 0.65 to 0.85, and mean R_{rand} values from 0.19 to 0.31 (III, Supplementary material, Table S2). In order to have good resolution of the ER stain, we had to use a different fixing and staining method than for lysosome visualisation. The ER method was not efficient enough for the lysosome staining, thus the values for wtMAN2B1 versus PDI are missing.

5.2.2 The homology model for human MAN2B1

Since human and bovine MAN2B1 share 80 % sequence identity, the bovine structure (24) is an excellent template for modelling the structure of the human enzyme. The homology model for human MAN2B1 comprises an N-terminal seven stranded active site α/β-domain connected by a three helix -bundle to three β-domains (Figure 2). The bovine structure consists of seven peptides (Figure 2) separated by proteolytic sites, which we modelled in human MAN2C1. Thus the amino acids missing from the bovine proteolytic sites are missing also from the human model. Altogether, the sequence covering the template structure is 84% identical to the human MAN2B1 sequence.

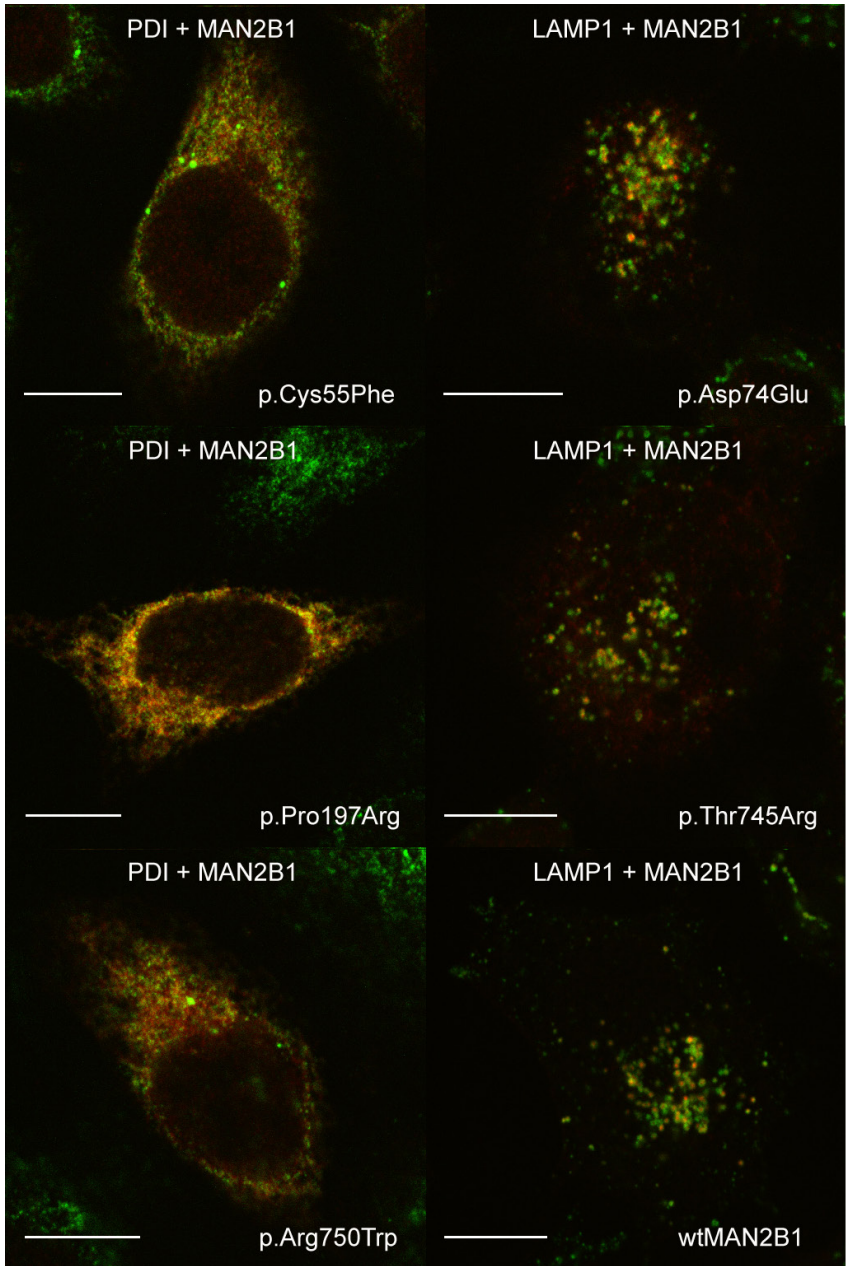

Figure 5. Intracellular localisation of α-mannosidosis associated MAN2B1 variants and wild type MAN2B1 in HeLa cells. Overlay images for MAN2B1(red) and for the ER protein PDI or the lysosomal membrane protein LAMP1 (both in green). The variant p.Arg750Trp retains in the ER (22), whereas the wild type (wt) MAN2B1 is in lysosomes. Scale bar corresponds to 10 μm.

The functional sequence for the human MAN2B1 model initiates after a signal sequence at amino acid residue G51, and is 962 residues in length. Although the human MAN2B1 functional sequence is twelve residues longer than the bovine MAN2B1, all deviations

are on the surface loops or within the proteolytic sites. The model contains the four cysteine bridges C55-C358, C268-273, C412-C472 and C493-C501 (Figure 2) and the active site Zn^{2+}, since these features are conserved in the human and bovine enzymes and thus are likely to be important for structural integrity. For the eleven glycosylation sites in the human MAN2B1 sequence (Figure 2) eight are conserved in the bovine protein. These were however excluded from the model due to incomplete structural data.

5.2.3 Consequences of the α-mannosidosis mutations on the MAN2B1 structure

Amino acids are not equally affected by α-mannosidosis missense mutations. Glycines were affected in 5 of the studied 29 missense mutations, leucines in 4/29 and arginines in 4/29 (III, Table 1). Overrepresentation in the disease mutations is not only due to their codon abundance (Gly has 4, Leu and Arg have 6), but also due to the properties of these amino acids. Glycine is the smallest amino acid because it is missing a side chain. The glycine substituting mutation therefore increases the residue volume, which may interfere with the packing of dense areas in the protein. Mutations on glycines often cause problems also because the side chain lacking glycine is important in providing conformational flexibility in turns and loops. Arginine has a positive charge and it can form ionic or hydrogen bonds. Due to this interaction capacity, arginines are commonly involved in substrate binding and the stabilisation of the interfaces inside or between proteins. Of the mutated arginine positions, two locate in the MAN2B1 interior, p.R202P close to the active site and p.R916C or p.R916H on the interface between the β3 and α/β-domain (Figure 2). The interface mutations are deleterious, and the protein is not detected beyond the ER (III, Table 1). The better tolerated mutations p.R229W and p.R950P are closer to the protein surface, and these variants enter lysosomes (III, Table 1).

Among the amino acids resulting from mutations, leucine, arginine and proline are the most severe ones and also overrepresented in the α-mannosidosis variants. Mutations result in leucine in 4 of the 33 variants, arginine in 5/33 and proline in 6/33 variants (III, Table 1). The arginine and proline variants tend to remain in the ER (III, Table 1), which indicates folding problems. Insertion of the long and charged arginine residues into the protein interior is complicated, because the stability of the fold demands that the arginine charge is involved in specific interactions. Most of the proline variants remain the ER (III, Table 1). The proline side chain forms its ring by binding to a backbone nitrogen, thus preventing the nitrogen from forming other interactions to stabilise the secondary structure. The rigidity induced by the ring prevents proline from adopting conformations available for other residues.

Disulphide bridges provide structural stability for proteins in harsh conditions such as in lysosomes. The cysteine mutations p.C55Y and p.C501S remove the two disulphide bridges, in which these residues are involved. The disulfide bridge C495-C501 stabilises a turn, which has the conserved glycosylation site, N497, in mammalian MAN2B1 proteins. The glycan in this site is exceptionally well-ordered in the bovine MAN2B1 crystal structure, suggesting a special role in folding, transport recognition

or enzyme function (24). However, the mutation p.C501S is surprisingly well-tolerated and transported to lysosomes (III, Table 1). The mutation deleting the disulphide bridge C55-C358 has more severe consequences, since the variant is not detected beyond the ER (Figure 5 and 6; III, Table 1). In the mature protein this disulfide bridge is located near the N-terminus, in the area with a special knot-like structure (III. Figure 4A), which indicates that the disulfide linkage has to form after the protein has been folded. Mutations in this area are deleterious, since in addition to the disulphide bridge-breaking p.C55F, the variant p.L352P, in which the mutation weakens the hydrogen bonding of the β-strand (III. Figure 4A), is retained in the ER (III, Table 1).

The MAN2B1 active site is on the protein surface (24), and hence should structurally tolerate mutations better than the core. Consistent with this assumption, the active site MAN2B1 variants p.D74E (Figure 5), p.D159N, p.H200N or L, p.P379L, p.G451C and p.V457E are destined to lysosomes (III, Table I, Figure 4B). The residue D74 participates in Zn^{2+} coordination, and replacing aspartate with the positively charged but shorter side chain of glutamate in p.D74E changes the metal binding site (Figure 6) and obviously the catalytic properties of the enzyme. H200 stabilises the active site residue R220 and hydrogen bonds to E160. The mutations are deleterious for hydrogen bonding capacity: p.H200N is left able to bind only one hydrogen atom and p.H200L is completely nonpolar. Thus these mutations destabilise the area. P379 initiates the loop consisting of amino acids 380-388, the potential substrate binding area.

The active-site variants p.P197R (Figure 5 and 6), p.R202P, p.S453F and p.S453Y (III, Figure 4B) are missing from lysosomes in HeLa cells but destined to lysosomes in COS-7 cells (III, Table 1). All these mutations introduce large changes in the residue volume. Replacement of S453 with tyrosine or phenylalanine inserts a large residue into the loop connecting helices in the 3-helix bundle, and due to the residue volume these variants are more severely affected than the adjacent p.G451C (III, Table 1). Since this loop connects to the active site, the insertion of larger residues could shift the position of the residues involved in substrate coordination, catalysis (W77, D447 and R823) or metal binding (H446). This area is also affected by another mutation, the variant p.814_815dupHR results in a histidine-arginine -duplication on the β-strand preceding a loop in the potential substrate binding area (III, Figure 4B).

Mutations resulting in p.Y99H and p.A95P locate on the interface of the MAN2B1 dimer (III, Figure 4C). Y99 participates in dimerisation by hydrogen bonding to residues Q98' and N231' on the other monomer. The dimer interaction is probably lost in p.Y99H, although the variant is still transported to lysosomes (III, Table 1). A95 initiates an α-helix on the dimer interface region. This is affected in p.A95P due to the proline ring introducing mutation, and the variant is retained/remains in the ER (III, Table 1).

The C-terminal e-peptide domain, formed by two proteolytically generated peptides, is crowded with α-mannosidosis mutations (III, Figure 1). The function of this domain is unknown, but it folds to the back of the protein and anchors to the rest of the structure by the initiating β-strand in the β1-domain and an elongated stretch on the surface of the active site domain (Figure 6, (24)). All the e-domain variants have difficulties

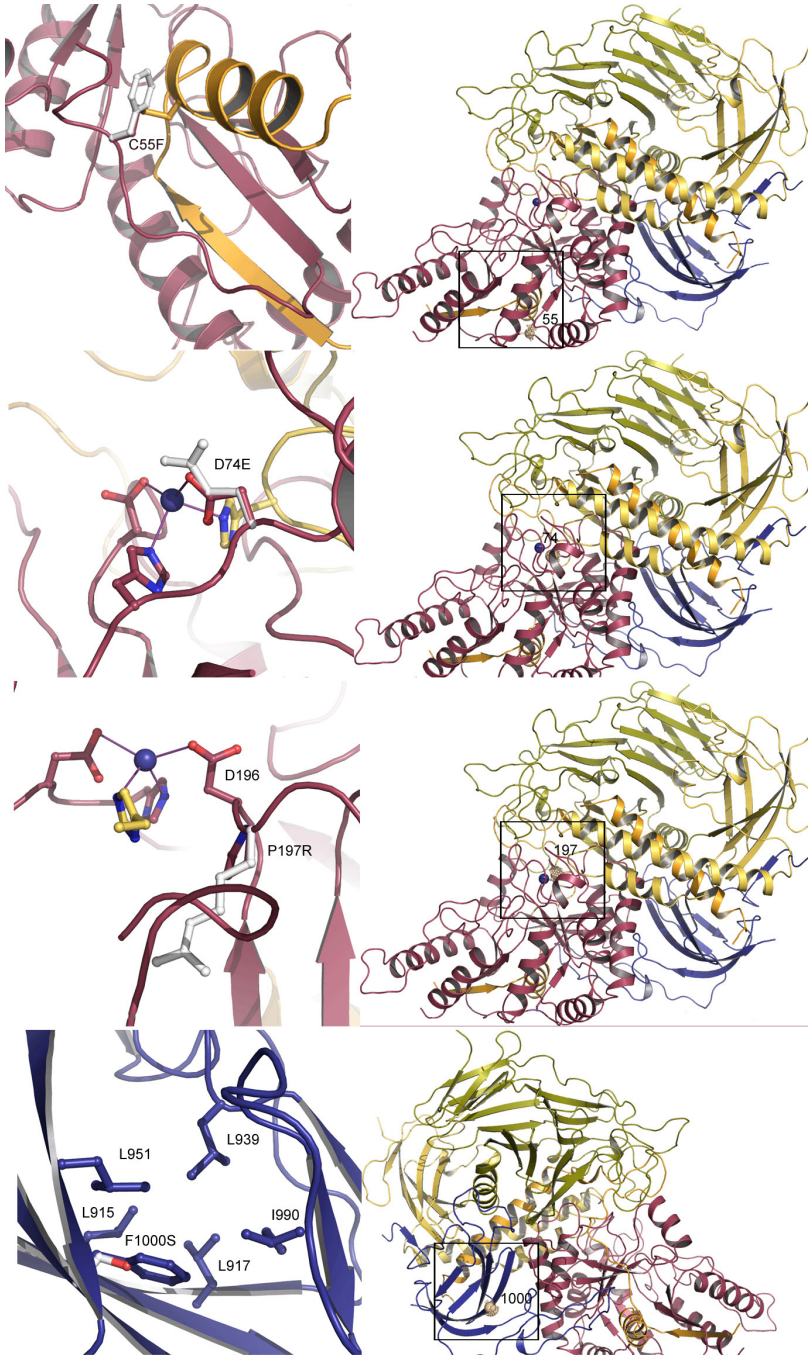

Figure 6. α-Mannosidosis associated mutations in the human MAN2B1 homology model. The polypeptide is colored according to proteolytic peptides in the bovine MAN2B1. The a-peptide containing the active site barrel is in red, peptides 'b', and 'c' forming the three helix bundle and first small β-domain are in orange and yellow, the large β-domain 'd' in green and the 'e'-peptide domain in blue. The overview of the MAN2B1 fold shows the site of the mutation in the MAN2B1 structure. In the detailed picture, the mutated residue is in white and the wild type residue in color. Zinc is in blue.

in exiting the ER (III, Table 1), thus this domain seems to be important for the sound structure of MAN2B1.

5.2.4 The mutation data is available through the α-mannosidosis database

The results of the α-mannosidosis variant characterisation on intracellular transport, extracellular secretion and proteolytic maturation form part of the novel α-mannosidosis database. In addition to these results, the database covers other relevant aspects in the disease: sequence data on mutations, their allelic combinations, remaining enzyme activity of the MAN2B1 missense mutation variants, effects of amino acid changes for the protein structure and patient data with clinical descriptions. All functions can be accessed from the home page by tabs leading to *Mutations*, *Experimental data*, *Patients and genotypes* and *Clinical data.* The home page introduces the database and the related EU-projects. It provides short descriptions on disease characteristics, genetics, the enzyme and mutations in the *MAN2B1* gene and links to further information in public databases. The home page also announces the current number for mutations, patients and clinical observations and dates for the latest updates.

The *Mutations* section lists all currently known 124 mutations, which are found in α-mannosidosis patients. The default arrangement of the mutations is based on their position in the MAN2B1 amino acid sequence and both the DNA base and amino acid changes are given in addition to exon/intron locations on the *MAN2B1* gene. Mutations are linked to further details in the database, such as experimental data, references to literature and patients carrying the mutation. A search tool allows enquiries with different parameters from the mutation data.

The table in the *Experimental data* section lists missense mutations, which have been individually expressed in mammalian cell lines. The MAN2B1 variant characterization includes α-mannosidase activity, level of intracellular processing, lysosomal transport and extracellular secretion of the variant protein. The α-mannosidase activity is measured from cell lysates and presented in a column diagram. The diagram also shows the activity of the wild type MAN2B1 and vector pcDNA3.1, which define the range of the enzyme activity. Intracellular proteolytic processing is shown with western blotting from COS-7 cells. The table column for 'Secreted into medium' is linked to another set of western blots, which show if the full-length form of the variant is secreted into the culture medium. The column for 'Intracellular localization' links into confocal microscopy images of immunolabelled HeLa cells expressing the individual MAN2B1 variants. MAN2B1 is costained with either a lysosomal or ER marker, to display the cellular localisation. The final column describes the effects of each mutation on the 3D model structure of the MAN2B1 protein and shows the location of the mutation in the overall protein structure.

Patient data is listed in a table which has information on sex, age and country of origin. In addition, it links to information on clinical data, when available, affected siblings and mutations on both genetic alleles, at the DNA and protein level. This page also shows patient statistics on sex, age and availability of mutation and clinical data. The

geographical distribution of patients in the database is shown in an interactive world map, which displays the amount of the database included patients in different countries.

The page *Clinical data* allows the user to search the clinical data by using the patient identification code number. The search will retrieve a column diagram representing 27 clinical parameters. These parameters describe both specific features and general issues in α-mannosidosis and the column diagram shows if the patient is diagnosed positive or negative for each individual feature.

6. SUMMARY OF THE DISCUSSION

6.1 A more detailed view on the cellular role of NAM (I, II)

A cobalt-dependent α-mannosidase activity was described in different animal tissues (118, 130). This activity was associated with NAM (95, 131-133), which was suggested to be involved in the cytosolic metabolism of free OS (10, 11). The details of NAM's cellular role still remain obscure, including how NAM participates in free OS metabolism, if has it any connection to N-glycosylation, what kind of cell biological effects this enzyme has, and to which cellular compartment it localises.

6.1.1 NAM is important in free OS metabolism

To produce adequate amounts of human NAM for biochemical and 3D structure analyses, we chose *P. pastoris* as the expression host after preliminary trials in mammalian, insect and yeast cells (data not shown). Although all these expression hosts were capable of expressing NAM, we selected *P. pastoris* due to good NAM expression levels and efficient up-scaling possibilities for recombinant protein production in a biocultivator.

The affinity tag allowed us to minimise the purification steps and to avoid the reported instability of NAM at high salt concentrations and especially during final purification steps when gel filtration was applied (119). We selected cobalt as the chelating metal, because the atomic absorption spectrum of the hen NAM suggests that the enzyme contains cobalt (133). Our strategy was to use the expected active site cation, and thus minimise the risk of ligand heterogeneity in cation analysis and crystallisation trials.

My work describes the characterisation of the purified human NAM both with the high-mannose type and artificial substrates. Man_9-substrates were chosen, because they form the lengthiest type of the possible natural substrates, which are released into the cytosol from the ERAD-targeted proteins. Furthermore, human NAM had not been characterised with Man_9-substrates. Trimming of $Man_9GlcNAc$ by NAM led to the main product $Man_5GlcNAc$ (I, Figures 3 and 4). NAM hydrolysis on $Man_9GlcNAc_2$ was limited to the isoform M8C (Figure 1; I, Figure 4), suggesting that the reducing end structure with the single GlcNAc is important for NAM hydrolysis. Similar results have been obtained with NAMs from different organisms or from α-mannosidase analyses of the cytosolic fraction of cells (93-96, 133). The selectivity towards the reducing end composition suggests that neither the N-glycans attached to proteins nor dolichol linked oligosaccharides would serve as substrates for NAM. The hydrolysis results for human NAM with the Man_9-substrates together with the pH-optimum of human NAM suitable for a cytosolic enzyme, propose that NAM is the enzyme responsible for trimming the free OS in the cytosol but that it needs PNGase and/or ENGase action for exposing the single GlcNAc at the reducing end.

The characterisation of the oligosaccharide content in the NAM overexpressing cells supported the idea of NAM being an important enzyme in cytosolic free OS catabolism.

The main free OS pool in the HeLa-NAM cells consisted of $Man_{2-4}GlcNAc$ instead of the $Man_5GlcNAc$ seen in wild type HeLa. Most likely, the shorter free OS species are effectively transported to lysosomes in wild type cells (98, 99), but in HeLa-NAM the overexpression of NAM produces such high amounts of these shorter species that the lysosomal transporter capacity is exceeded and $Man_{2-4}GlcNAc$ remain in the cytosol. The reason why an *in vitro* test with purified NAM produces mainly $Man_5GlcNAc$ and only small amount of $Man_4GlcNAc$ is probably due to different conditions in the natural cellular environment compared to those *in vitro.*

The members of the GH38 family bind cationic metals. The crystal structures for both the fruit fly Golgi α-mannosidase and the bovine MAN2B1 have zinc in the active site (24, 116), whereas NAM homologues have been described as cobalt activated enzymes (11, 95, 96, 133). The hen NAM is a tetrameric enzyme that contains two cobalt atoms, and incubation with cobalt doubles its metal content (133). Without cobalt, human NAM only released a single mannose from the C-branch of $Man_9GlcNAc$ (Figure 1). In the presence of cobalt, NAM trims four mannose residues (97). Surprisingly, we found that Fe^{2+} was an even stronger activator for NAM than Co^{2+}. Cation tests with $Man_9GlcNAc$ and PNP-α-man yielded similar results, thus the cations could be divided into groups of 'activating', 'null effect' and 'inhibiting'. The only class switcher in the tests with different substrates was Zn^{2+}, which was capable of removing only a single mannose from $Man_9GlcNAc$, but had as high a hydrolysis rate as Co^{2+} with PNP-α-man (I, Table I and II). This suggests that all the details for the enzymatic mechanism are not necessarily revealed with a simple artificial substrate.

Confocal microscope images of immunofluorescence labelled NAM and the ER marker EGFP-kdel suggested that NAM is cytosolic and absent from the ER (I, Figure 6). Due to covering properties of the cytosolic staining, a simultaneous visual detection of the targets in other organelles may be unreliable. Therefore, we calculated the statistical correlation of the pixels of the optically sectioned NAM and EGFP-kdel images using the Costes algorithm (164), which indicated that NAM is not present in the ER. Absence from the ER is compatible with our findings that NAM does not possess a signal sequence and remains soluble in the HeLa-NAM subcellular fractionation.

Since the punctuate immunofluorescent staining of NAM did not result from NAM appearing in lysosomes or autophagosomes, we investigated the mobility of NAM by fluorescence recovery after photobleaching (FRAP; paper I). In the FRAP experiment, the recovery of the fluorescence of the NAM-EGFP fusion after a laser pulse in live cells was compared with the recovery of a soluble cytosolic protein, native EGFP. Due to the different sizes of the two molecules, NAM-EGFP recovery is expected to be 2.74-fold slower than the recovery rate of EGFP. The observed recovery rate of NAM-EGFP was, however, slower than expected, 3.32-fold. The FRAP recovery curve demonstrates (I, Figure 7) that the recovery of NAM-EGFP has two phases: the majority of the molecules retrieve rapidly but for a subset of NAM-EGFP population, the retrieval is slower. The rapid recovery most likely represents free soluble molecules, whereas the slower recovery represents molecules with limited mobility due to interactions with cytosolic structures.

My work also showed that NAM colocalises with proteasomes. This is in agreement with the suggested role for NAM in the trimming of N-glycan moieties of the ERAD substrates which have their peptide moieties degraded in proteasomes. The localisation result is similar to the localisation of PNGase and ENGase, which are also involved in N-glycan catabolism; in addition that both these enzymes are cytosolic (79, 87, 178), PNGase colocalises and interacts with proteasomes (86). It is possible that the proteasome colocalisation explains the membrane-associated NAM in the HeLa-NAM subcellular fractionation and earlier studies (14, 15). Due to the resolution limit of the confocal microscope, colocalisation does not prove that the colocalised molecules would physically or chemically interact. Thus the relation of NAM to proteasomes needs to be determined in the future with methods other than confocal microscopy. For probing for the weak interactions, proteomics either with stable isotope labelling with amino acids in cell culture (the SILAC method) or a yeast two hybrid method could be used.

6.1.2 NAM has an impact on N-glycosylation and ERAD

Paper II describes the effects of NAM overexpression in living cell and clarifies the physiological role of NAM. The enhanced NAM expression resulted in an elevated cellular concentration of M6P. A similar situation is described in the cells of the patients with congenital disorder type Ia (179). These patients have increased M6P concentrations due to deficient activity of phosphomannomutase 2, which converts M6P to mannose-1-phosphate (180). The proteins in the patient cells are hypoglycosylated, which is possibly caused by the increase in M6P (179). The NAM overexpressing cells have decreased concentrations of UDP-Glc. Because glucose metabolism is irreversibly linked to M6P metabolism (181), NAM overexpression leads to a reduced UDP-glucose supply to be used in N-glycan precursor synthesis and finally results in the addition of truncated, unglucosylated N-glycans on proteins, and underglycosylation. Since both UDP-Glc and M6P are precursors for DLO, their biased cytosolic concentration likely leads to the biosynthesis of a truncated DLO precursor, which is lacking its glucose residues. This is exceptional, because in normal conditions OST only adds mature precursor molecules from the DLO precursor onto nascent peptides (7). In HeLa-NAM we see underglycosylation of the known ERAD substrate NHK and the cell membrane protein β1-integrin, and enhanced ERAD. Underglycosylation and missing glucose residues may have various direct physical effects on protein folding, and the unglucosylated proteins may also have difficulties in entering the calnexin/calreticulin cycle properly. Deglucosylation does not however totally block proteins from the calnexin-calreticulin cycle: the single glucose residue detected on protein N-glycans (II, Figure 6A) most likely results from the action of UGGT during the calnexin-calreticulin cycle.

6.2 Molecular consequences of α-mannosidosis missense mutations (III, IV)

6.2.1 Mutation classification according to MAN2B1 processing and intracellular location

Confocal imaging detects on the single cell level which MAN2B1 variants are retained in the ER and which are transported into lysosomes. Another indication for successful MAN2B1 transport into the endosomal-lysosomal compartment is the proteolytic processing of the native polypeptide. Paper III shows the analysis of the wild type MAN2B1 processing: it produces bands for the peptides abc- (visible at 65 kDa), ab- (at 46 kDa) and de-peptides (at 38 kDa) (III, Figure 2). Further processing products are difficult to identify due to the small and overlapping size of the peptides. During overexpression, MAN2B1 is also secreted into the culture medium as a full-length polypeptide (III, Figure 2).

Analysis of the intracellular localisation, proteolytical processing and secretion of MAN2B1 α-mannosidosis variants allowed the categorisation of α-mannosidosis associated mutations into subtypes. Mutations, which prevent transport to lysosomes, lead to failure in the proteolytic processing and secretion of the enzyme (III, Table 2; group 1). All these ER retaining variants involve mutations on the structurally important residues arginine, proline, cysteine or glycine. The result that half of the mutations affect the e-peptide emphasises the structural importance of this domain.

The majority of the analysed α-mannosidosis variants are transported to lysosomes, secreted into the medium and proteolytically processed, although they produce fainter bands on western analysis than wild type MAN2B1 (III, Figure 2 Table 2; group 2). More than half of the affected residues in this group involve in the active site (III, Table 1), and the heterogeneity of the amino acids involved in the mutations is greater than among the ER-retaining variants. A few variants in group 2 show cell line specific localisation (III, Table 1): although they are absent from the lysosome in HeLa cells, they are lysosomal in COS-7 cells. This difference is possibly due to the expression level, which most likely is lower in HeLa than in the efficiently overexpressing COS-7 cells. Distinctive from the ER-retaining variants, some of the lysosomal variants show residual α-mannosidase activity, when they are heterologously expressed (28).

A subset of the unprocessed ER-retaining variants behave unconventionally and are extracellularly secreted (III, Table 2, group 3). The secretion suggests that these variants are capable of exiting the ER, although they are not visible in lysosomes. An explanation for this could be that the variants are initially transported to lysosomes, but due to poor stability in harsh lysosomal conditions, they are rapidly hydrolysed. Thus we can only detect the variant molecules that are trapped in the ER-quality control due to unsuccessful folding or overexpression or both. Another unconventional set of variants is processed but not secreted (III, Table 2, group 4). This might be due to low expression levels, so that all MAN2B1 molecules are transported to lysosomes and there are no excess molecules to be secreted. Likewise, the folding problems in the ER could lead

to a situation where only a small number of molecules exits the ER and is not secreted, because the lysosome transport machinery is not overloaded.

6.2.2 Prediction of the molecular pathogenicity of MAN2B1 missense mutations

Although the missense mutations spread through the MAN2B1 sequence, a few correlations with the molecular consequences and the site of the mutation exist. The b- and e-peptide are more crowded with mutations than are other peptides (Figure2). In the MAN2B1 structure, the folded b-peptide completes the active site and connects it to a three-helix bundle and the conserved glycan N497, whereas the e-peptide folds into a flat surface domain opposite to the glycan N497. This e-peptide seems to be vitally important, since nearly all mutations affecting it lead to severe folding problems and the disease variants are unable to exit the ER. On the contrary, mutations affecting the active site α/β -domain seem to be less harmful at the molecular level, since the analysed cell biological features of most of these variants are similar to wild type MAN2B1.

Paper III describes the proteolytic processing of nine benign MAN2B1 missense mutations. These sites for single nucleotide polymorphism (SNP) provide a comparison tool with pathogenic mutations. Although the SNPs affect the same amino acid species as disease mutations, nearly all of the SNP amino acids situate on the protein surface (III, Table 3). Surface residue changes are often better tolerated than are mutations which affect the protein interior.

The experimental analysis on α-mannosidosis variants evaluated if a mutation allowed or disrupted normal intracellular transport, but it did not measure the quantitative pathological effect of the mutation. Pin-pointing genotype-phenotype correlations or phenotype correlations between the presented four mutation subclasses is currently not possible, since clinical phenotyping is available only for a limited number of missense mutations (28). The four mutation subclasses are also unequally represented in the clinical phenotyping data, thus the patient data needs to be expanded in order to have a statistically meaningful set of information.

6.2.3 The database for α-mannosidosis is an effective source for disease information

Information on α-mannosidosis-associated mutations and patient descriptions have so far been scattered in the original publications. The α-mannosidosis database covers the important aspects of the disease and brings them available via a single internet site. Compiling all information in one site is especially important, since α-mannosidosis is a rare but widely spread disease.

Not only the mutation data but also the effects of mutations on the MAN2B1 enzyme structure and function and descriptions of patients and their clinical features is found on the site. Mutations which result in truncated forms of the MANB1 enzyme, are recognized by the ER quality control mechanism and degraded, thus predicting the molecular and cell biological consequence of these mutations is fairly straightforward. Missense mutations include a variety of failures in function, which lead to impaired

α-mannosidase activity. The database provides an efficient search tool, which enables the extraction of missense mutations and the experimental and structural data that is linked to them. This greatly widens the perspective compared to a situation where only the sequence change is known. Thus the database provides a large set of experimental data to support the understanding of the molecular mechanism of the missense mutations. In addition, the database combines this information to show the second allele mutation and the resulting clinical phenotype type of the patient, and provides a broader view of the physiological consequences of each mutation and MAN2B1 haplotype at level of the individual.

The central aspect in α-mannosidosis database is that it will not only provide a wide range of molecular information for pathophysiological studies and patient descriptions for clinical use, but it will improve interactions between the clinical and molecular sciences involved in the disease. Availability of the clinical data will be beneficial for the molecular scientists, who aim to solve genenotype-phenotype correlations, consequences of accumulation of the unhydrolysed oligosaccharides and attempt to develop specific treatments for α-mannosidosis. For physicians, the molecular data will in the future provide tools for personalised treatment options, depending on the molecular pathophysiology of the patient.

7. CONCLUDING REMARKS AND FUTURE PROSPECTS

The thesis describes the characterisation of two GH38 α-mannosidases, human NAM and MAN2B1, which express ubiquitously in different organisms and cell types and are involved in the metabolism of N-glycans. Both the *in vitro* substrate characterisation of the purified recombinant NAM and the *in vivo* analysis of the free oligosaccharides during NAM overexpression suggest that this enzyme is important for the hydrolysis of cytosolic free OS. As presented in the paper I, NAM hydrolyses $Man_9GlcNAc$, yielding $Man_5GlcNAc$ as the main product. The latter oligosaccharide is a common free OS in the cytosol, which we have shown in HeLa cells (II). Cytosolic substrates for NAM are likely released from the misfolded ERAD substrates by the enzymes PNGase and ENGase. Overexpression of NAM in HeLa cells increases the total amount of free OS, the effect reversed by the GH38 inhibitor swainsonine.

Since the biosynthesis and catabolism of N-glycans occur in defined cellular compartments, it is important to determine the location of the enzymes involved in order to understand their physiological roles in these processes. We showed that NAM it is a cytosolic enzyme with a subpopulation of molecules colocalising with proteasomes. Colocalisation with proteasomes is in accordance with the proposed role for NAM in the hydrolysis of N-glycans that are released from the proteasome-destined target proteins of ERAD. According to localisation studies NAM is absent from the ER and Golgi. The present knowledge (summarised in 2.2) of the ER-located mannosidases suggests that NAM is not needed in the ER but mannose trimming in this organelle is performed by ER mannosidase I and EDEMs. Thus, NAM would not be a ER II mannosidase, as has been previously suggested (15). If NAM is present only in the cytosol, underglycosylation of the secreted proteins in the HeLa-NAM cells is not due to NAM action in these organelles, but is supposedly caused by the NAM-induced change in the cytosolic concentration of the N-glycosylation precursor molecules, mannose-6-phosphate and UDP-glucose. The underglycosylation of the secreted proteins during the NAM overexpression evokes a question: is NAM expression linked to any of the disease associated glycosylation changes? The extracellular effects of siRNA inhibition of NAM on cancer cells have been reported (17, 18), but it will be interesting to investigate NAM expression profiles in cancers or protein folding diseases. At least the regulation of NAM expression seems to be important for correct N-glycosylation.

The clearance of the free OS from the ER is possibly important since soluble glycans might disturb the glycan-dependent processes of protein folding, quality control, ERAD and transport. The importance of the cytosolic clearance probably serves for recycling the building blocks. It also helps to maintain cellular homeostasis and allows effective degradation of ERAD substrates in proteasomes. It is currently not known if there are pathophysiological states involved in free OS metabolism linked to NAM and, what would be the consequences of the complete loss of NAM activity due to mutations in the NAM gene. And at the organism level, what would be the phenotype for a NAM-deficient mouse model? Since NAM and MAN2B1 contribute to the same pathway, it would be worth investigating if NAM activity is linked to any of the disease phenotypes seen in

α-mannosidosis. For more detailed information on NAM's physiological role, it would be useful to investigate the interactome of NAM by proteomics.

The yeast expression system allows the production of adequate amounts of homogenous human NAM for crystallisation and finally 3D structure determination, which would be a one step forward in clarifying the function of NAM. The 3D structures for mammalian GH38 enzymes are only known for bovine MAN2B1. Since NAM has low sequence homology with lysosomal and Golgi α-mannosidases, the currently available structures do not provide information that could be reliably applied to NAM. The 3D structure would make it possible to better understand the substrate and inhibitor specificity of NAM, and comparison with the known GH38 structures would help to understand adaptations to different cellular locations and the structures of the additional domains.

The study of α-mannosidosis missense variants combined cell biological and structural analysis. When judged by transport to lysosomes, proteolytic maturation and secretion, the variants behaved differently and we could divide them into groups representing problems in folding, lysosomal stability and enzyme function. The structural analysis revealed tendencies in the amino acid changes: the mutations which lead to large changes in the side chain volume or affected amino acids that are important for folding, lead to serious molecular consequences. The areas, which do not tolerate movement, are especially sensitive for the effects of these types of mutations. Additionally, we concluded that amino acid changes in the domain or dimer interphases often lead to folding problems, whereas the non-pathogenic mutations commonly affect the amino acid residues, which are exposed on the protein surface or are at least located on the surface structures and regions allowing structural flexibility. Our description of different types of missense mutations could help to predict pathogenic mutations and their molecular effects also in other lysosomal proteins when the 3D structure of the protein is known. For this purpose, a similar cell biological study should be conducted on other lysosomal proteins linked to LSDs, such as acid α-glucosidase (gene defect in Pompe disease, OMIM #606800 and #232300) or α-galactosidase (Fabry disease, OMIM #301500). The characterisation of disease-associated protein variants would also increase the information on normal lysosomal proteins: which features make them soluble and stable in harsh lysosomal conditions or are crucial for their successful folding in the ER and transport in the lysosomes.

Although chaperone therapy is not yet applicable as a treatment for α-mannosidosis, the variants that we found to be retained in the ER due to folding problems or the variants which have stability problems in lysosomes could be possible targets for this therapy. Chaperone testing for Pompe disease variants has been conducted with 1-deoxynojirimycin, which had a stabilising effect on several missense variants, which showed increased variant activity, proteolytic maturation and transport to lysosomes (182). Similar effects should be analysed also for wild type MAN2B1 and on α-mannosidosis missense variants with potential chaperones. The first step towards the therapy would be screening for the potential chaperones among the small molecules, which inhibit GH38 α-mannosidases. The molecules with inhibitory properties have been tested on Golgi II α-mannosidase in particular (183), because this enzyme

is a target for cancer therapy (116). Recent findings also suggest that an increase in intracellular calcium has a positive effect on the folding of the α-mannosidosis variant p.P356R (184), thus the effects of Ca^{2+} also on other variants would be worth investigating. One more strategy for treating α-mannosidosis with small molecules would be the administration of zinc, which increased α-mannosidase activity in the cell extracts of two Palestinian siblings with α-mannosidosis and control subjects (185) but did not yield positive results in later trials (186). Due to the structural knowledge of the active site amino acids involved in metal binding and coordination of Zn^{2+} (24), the patients with the mutations that possibly cause weaker metal binding could benefit from the supplementary zinc.

In the future, this study may also help to answer a fundamental problem: does a mutation with serious molecular consequences also lead to serious clinical symptoms. To find out genotype – phenotype correlations in α-mannosidosis, the clinical typing must be available from a larger amount of patients carrying these missense mutations than it is now. A useful tool for this analysis would be the α-mannosidosis database. As presented here, it provides an updatable source for this rare disease and contains disease haplotypes, their frequencies, and geographical distribution, clinical information and compiles also the non-pathogenic mutations, SNPs. When the specific treatment options such as enzyme replacement therapy, gene therapy or chaperone therapy will hopefully be available, the clinical data could be updated with descriptions on the effectiveness of the therapy. Eventually this might help in tailoring the best therapy options for the patients once their mutations have been analysed.

8. ACKNOWLEDGEMENTS

This thesis study was mostly carried out in Department of Biochemistry and Food Chemistry, University of Turku. I am deeply grateful to Professors Jyrki Heino and Reijo Lahti for proving me excellent working facilities although the rest of our research group was for most of the time situated in the Institute of Biotechnology, University of Helsinki.

A very important person to me and my work has been my supervisor, Professor Pirkko Heikinheimo. It has been a great pleasure to learn from her how to do science. Pirkko has a great deal to do not only with my scientific career but also why I became involved in dog training. Thank you for counting on me and pushing me forward.

I am also grateful for Professor Mark S. Johnson, the head of the National Doctoral Programme in Informational and Structural Biology. Being a student in such a scientifically ambitious graduate school has been an encouraging experience. The ISB seminars in Lapland have been an excellent chance to get to know the science in the area of structural biology and bioinformatics in Finland. These events have also meant a chance to get together with the friends in the graduate school, thank you all for sharing the good moments.

The annual meetings with my follow-up committee have played a central role for the thesis process. I want to acknowledge Professor Reijo Lahti and Docent Tiina Salminen for fruitful discussions and giving new perspectives to my work.

I sincerely thank Docents Sakari Kellokumpu and Jani Saarela for critically reviewing the manuscript and Helen Cooper for revising the language. Professor Tommaso Beccari is greatly acknowledged for being the opponent.

I spent the first year of the thesis study in Northern Norway, in University of Tromsø. This year gave a good introduction to GH38 α-mannosidases, Norwegian life and tremendously beautiful nature. I would like to thank Professors Edward Hough, Ole-Kristian Greiner-Tollersrud and Øivind Nilssen for the opportunity to work there, Thomas Berg for the enthusiastic supervision and Hilde Monica Frostad Riise Stensland and Øivind Nilssen for collaboration with the MAN2B1 mutation characterisation. Special thanks go to Helle, Gry and Gaute for making my visit in Norway so enjoyable. I am also deeply grateful to a fruitful collaboration with Sandrine Duvet and Professor Rene Cacan in University of Science and Technology in Lille, France. Oligosaccharide analysis in Lille was an important step in NAM characterisation and the visits to Lille were pleasant experiences.

During these years I have had good colleagues in our group: Marika, Heidi Repo and Heidi Tuominen, Tiina, Wesley, Seija, Chiara, Elise: it has been wonderful to work with you. Special thanks' also to Professor Adrian Goldman's group, especially Esko, Ansko, Jack, Andrzej for listening to my presentations in the group meetings and giving feedback for my work.

I would also like to thank past and present colleagues and students in Department of Biochemistry and Food Chemistry for creating a friendly working environment. Special thanks go to Riikka and Anna-Mari; it was lots of fun to share a lab with you. I am also very grateful to Jani Sointusalo, Anu Hirvensalo, Satu Jasu, Jarmo Käpylä and Pekka Rappu for solving the problems with the laboratory equipment, administration or computers. Technical assistance with the imaging was invaluable for the thesis work: I would like to thank Jari Korhonen, Pasi Kankaanpää, Mika Molin and Kimmo Tanhuanpää and also Johanna Jokinen for helpful discussions regarding immunofluorescent staining.

There is also life outside the lab. I would like to thank the Academic hiking team: Johanna, Piia, Kaisa and Paula for sharing both the good and not-so-good sides of academic life in our long distance and short distance (mostly Pääskyvuori) trips. Thank you, Outi, for interdisciplinary discussions. No matter if it is an academic or a dog training problem, you always have a solution. I would also like to thank all other dear friends of mine and my family for a constant support. And of course, for Mikko, who has bravely carried his share of the burden of my thesis work.

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