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Family 47 α -Mannosidases in *N*-Glycan Processing

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Abstract

α -Mannosidases in eukaryotic cells are involved in both glycan biosynthetic reactions and glycan catabolism. Two broad families of enzymes have been identified that cleave terminal mannose linkages from Asn-linked oligosaccharides (Moremen, 2000), including the Class 1 mannosidases (CAZy GH family 47 (Henrissat and Bairoch, 1996)) of the early secretory pathway involved in the processing of *N*-glycans and quality control and the Class 2 mannosidases (CAZy family GH38 [Henrissat and Bairoch, 1996]) involved in glycoprotein biosynthesis or catabolism. Within the Class 1 family of α -mannosidases, three subfamilies of enzymes have been identified (Moremen, 2000). The endoplasmic reticulum (ER) α 1,2-mannosidase I (ERManI) subfamily acts to cleave a single residue from Asn-linked glycans in the ER. The Golgi α -mannosidase I (GolgiManI) subfamily has at least three members in mammalian systems (Herscovics *et al.*, 1994; Lal *et al.*, 1994; Tremblay and Herscovics, 2000) involved in glycan maturation in the Golgi complex to form the Man₅GlcNAc₂ processing intermediate. The third subfamily of GH47 proteins comprises the ER degradation, enhancing α -mannosidase-like proteins (EDEM proteins) (Helenius and Aebi, 2004; Hirao *et al.*, 2006; Mast *et al.*, 2005). These proteins have been proposed to accelerate the degradation of misfolded proteins in the lumen of the ER by a lectin function that leads to retrotranslocation to the cytosol and proteasomal degradation. Recent studies have also indicated that ERManI acts as a timer for initiation of glycoprotein degradation via the ubiquitin-proteasome pathway (Hosokawa *et al.*, 2003; Wu *et al.*, 2003). This article discusses methods for analysis of the GH47 α -mannosidases, including expression, purification, activity assays, generation of point mutants, and binding studies by surface plasmon resonance.

Overview

The biosynthesis of glycoproteins containing Asn-linked oligosaccharides is initiated by the transfer of a Glc₃Man₉GlcNAc₂ oligosaccharide from a lipid-linked precursor to nascent polypeptides in the lumen of the endoplasmic reticulum (ER) (Kornfeld and Kornfeld, 1985). After the glycan transfer, a series of processing steps occurs, wherein hybrid or complex oligosaccharides can be formed from the initial high-mannose precursor. The initial steps of this process involve the trimming of glucose residues by glucosidase I and II, enzymes that are localized in the ER. These enzymes are responsible for the excision of two of the three glucose residues to form the GlcMan₉GlcNAc₂ structure necessary for recognition by the lectin chaperones, calnexin and calreticulin. Further trimming by glucosidase II removes the final Glc residue to result in the release from calnexin/calreticulin interactions. Subsequent trimming of mannose residues from the precursor oligosaccharide is a prerequisite for the maturation of *N*-glycans to hybrid and complex-type structures, and in mammalian cells these cleavage reactions are accomplished by the action of ERManI, members of the GolgiManI subfamily of enzymes, and an α 1,3/ α 1,6mannosidase in the Golgi complex, termed Golgi α -mannosidase II (Moremen, 2000). ERManI and the GolgiManI isozymes are Class 1 (GH47) α -mannosidases, whereas Golgi α -mannosidase II is a Class 2 (GH38) mannosidase and has been reviewed elsewhere (Moremen, 2000, 2002). Alternative path-ways for high-mannose glycan maturation have also been proposed involving enzymes termed ER α -mannosidase II and endo α -mannosidase, which can trim Man₉GlcNAc₂ to Man₈GlcNAc₂ and Glc₃₋₁Man₉GlcNAc₂ to

Man₈-GlcNAc₂, respectively. These latter enzymes have also been previously reviewed (Spiro and Spiro, 2000; Weng and Spiro, 1996).

Class 1 α -mannosidases recognize and cleave only α -1,2-linked mannose residues from the Man₉GlcNAc₂ high-mannose precursor, but each of the subfamilies has unique specificities for recognition of terminal α -1,2-mannose residues in the high-mannose structure. The ERManI subfamily contains a single member in all eukaryotes that have been examined, and the enzyme primarily cleaves a single mannose residue from Man₉GlcNAc₂ to produce a unique Man₈GlcNAc₂ isomer (Man₈ isomer B; Fig. 1). ERManI is the first mannosidase to act in this pathway, and it has been suggested that it mediates quality control in the ER by creating a specific glycoform that is recognized by members of the putative degradation family of lectins, termed the EDEM proteins. The GolgiManI subfamily of enzymes comprises three isoforms in mammalian organisms: Golgi-ManIA (Lal *et al.*, 1994), GolgiManIB (Herscovics *et al.*, 1994), Golgi-ManIC (Tremblay and Herscovics, 2000). The substrate specificities of each of the isoforms are similar, and they are all complementary to the action of ERManI (Gonzalez *et al.*, 1999). Thus, the GolgiManI isoforms can efficiently trim the Man₈GlcNAc₂ isomer B down to Man₅GlcNAc₂, but they poorly cleave the central branch mannose residue that is the target of ERManI action (see Fig. 1) (Lal *et al.*, 1998). Subsequent transfer of a terminal GlcNAc residue by GlcNAc transferase I and mannose trimming by Golgi α -mannosidase II result in the formation of the GlcNAcMan₃GlcNAc₂ intermediate that acts as the committed step in the synthesis of complex-type *N*-glycans.

The EDEM subfamily of proteins is composed of three isoforms, and two members (EDEM1 and EDEM2) do not appear to have catalytic activity. Recent data suggest that the third isoform (EDEM3) may have glycosidase activity toward high-mannose oligosaccharides (Hirao *et al.*, 2006). All three of these mannosidase homologs have been shown to participate in the targeting of misfolded glycoproteins from the ER to the cytosol for degradation by the ubiquitin-proteasomal system. The mechanism for this protein disposal function has not been determined, but it is thought that EDEM proteins act as lectins, using the same oligosaccharide binding site employed by the catalytic Class I α -mannosidases (Mast *et al.*, 2005).

ERManI was first isolated, characterized, and cloned from *Saccharomyces cerevisiae* (Herscovics, 1999), but subsequent cDNA cloning, expression, and characterization of the human ERManI homolog (Gonzalez *et al.*, 1999) have revealed similarities in structure and specificity between the fungal and human enzymes. Recombinant forms of human (Karaveg *et al.*, 2005; Vallee *et al.*, 2000a) and yeast ERManI (Vallee *et al.*, 2000b) have been expressed in large quantities in *Pichia pastoris*, and the structures of the catalytic domains of each enzyme have been determined by X-ray diffraction in the presence and absence of bound enzymatic products, small molecule inhibitors, and uncleavable pseudosubstrates.

Members of the GolgiManI subfamily of enzymes have been isolated from a variety of species sources (e.g., rat, rabbit, pig, and calf, reviewed by Moremen *et al.* [1994]), and recombinant forms of the human (Bause *et al.*, 1993) and mouse enzymes (Herscovics *et al.*, 1994; Lal *et al.*, 1994) have been expressed and characterized. Each of the members of the GolgiManI subfamily has been expressed in *P. pastoris*, and the structure of mouse GolgiManIA has been solved as a co-complex with a bound glycan enzymatic product (Tempel *et al.*, 2004). In addition, structures have been determined for the secreted GH47 α -mannosidases from *Penicillium citrinum* (Lobsanov *et al.*, 2002) and *Trichoderma reesei* (Van Petegem *et al.*, 2001), fungal enzymes that have substrate specificities similar to the GolgiManI enzymes.

The membrane topologies of the ERManI and GolgiManI subfamily members are similar, each containing a short cytoplasmic tail, transmembrane domain, linker region, and luminal catalytic domain (Moremen, 2000). The catalytic domains of both ERManI and GolgiManIA are both barrel-shaped ($\alpha\alpha$)₇ structures composed of two concentric layers of α helices containing a broad open cleft at one end of the barrel structure and a β -hairpin plugging the opposite end of the barrel. The oligosaccharide substrates bind to the open cleft, and extended interactions across the cleft provide specificity for insertion of glycan branches into the barrel core containing the enzyme active site. Structural data indicate that the cores of the catalytic pockets (+1 and -1 substrate-binding subsites) are conserved between the ERManI and GolgiManI subfamily members; however, outside of this area (>+1 residues), including the broad open clefts adjacent to the catalytic sites, the enzymes have distinct oligosaccharide binding sites that provide differences in branch specificities for these enzyme subfamilies (Tempel *et al.*, 2004). The energetic contributions of oligosaccharide substrate binding to the catalytic pocket and the broad open cleft were examined in a series of surface plasmon resonance binding studies between a catalytic mutant of human ERManI (E330Q) and a series of high-mannose *N*-glycan structures (Karaveg *et al.*, 2005). These studies revealed that the majority of the binding energy was contributed through interactions with the substrate residue in the +1 subsite (~52% of the substrate binding energy). Considerably less energetic contribution was provided by interactions with the -1 subsite residue (~21% of the binding energy). The remainder of the binding energy (~27% of the binding energy) was provided by substrate residues in the >+1 substrate binding subsites. Despite the relatively minor contributions of the >+1 subsite residues to the binding energy, the stereochemistry of the interactions in the broad substrate-binding cleft clearly provides the branch specificity for the selective cleavage of terminal α 1,2-mannose residues. Swapping of a key substrate-binding residue from GolgiManIA to ERManI led to broader specificity for glycosidase cleavage but poor catalytic efficiency and a significantly reduced binding affinity. Thus, the catalytic pockets and adjacent substrate-binding clefts for the Class 1 (GH47) α -mannosidases provide for the high-affinity and high-specificity substrate interactions that lead to the unique substrate recognition characteristics for the individual subfamilies. This chapter describes the methods for enzyme expression and characterization for wild-type and mutant forms of the Class 1 mannosidases that can be used as paradigms for similar studies in other enzyme systems.

Overview of Class 1 (GH47) Expression and Purification

Recombinant expression of wild-type and mutant Class 1 mannosidases in *P. pastoris* has proven to be an effective strategy for large-scale production of enzymes for biochemical and structural studies (Karaveg and Moremen, 2005; Karaveg *et al.*, 2005; Tempel *et al.*, 2004). The advantages of this expression system include the ease in generating recombinant constructs and transformants, the short-time course of induction, the high levels of protein expression, the ability to generate recombinant proteins that are secreted into the culture media for easy purification, and the ability to generate recombinant products that contain eukaryotic post-translational modifications (i.e., disulfide bond formation, *N*-glycosylation). We have generally used commercial expression vectors (Invitrogen pPICZ α series or pHIL-S1) that encode an NH₂-terminal *S. cerevisiae* α -factor signal sequence or *PHO1* secretion signal followed by a series of restriction sites. cDNA sequences encoding the COOH-terminal catalytic domains of the Class 1 mannosidases (with deletions of the NH₂-terminal cytoplasmic tails, transmembrane domains, and stem regions) have been inserted in-frame into the vectors, and transformants were generated in either Mut⁺ (X-33, GS115) or Mut^S (KM71H) strains. Following growth of cell mass on glycerol as a carbon source, enzyme expression was initiated by shifting to methanol as a carbon source and inducing agent for the AOX1 promoter. These transformants generate translation products that are targeted to the yeast secretory pathway and released into the culture media as potentially glycosylated

products. Both *S. cerevisiae* ERManI (Vallee *et al.*, 2000b) and mouse GolgiManIA (Tempel *et al.*, 2004) coding regions contain acceptor sequences for *N*-glycosylation (Table I) while human ERManI (Vallee *et al.*, 2000a) contains no *N*-glycosylation sites and is secreted as a non-glycosylated protein. In general, affinity tags have not been used for the generation of α -mannosidase recombinant constructs, since purification of the enzyme from the culture media is readily accomplished with two or three chromatography steps (see later). In recent studies, the mannosidase homology domain of human EDEM2 has also been expressed in *P. pastoris* using pPICZ α constructs. These latter constructs have been generated as epitope and/or His-tagged fusions at the NH₂-terminus of the α -mannosidase-homology domain, because previous data have indicated that COOH-terminal fusions yield in inactive ERManI constructs. A summary of α -mannosidase constructs generated for expression in *P. pastoris* is shown in Table I.

Generation of Class 1 α -Mannosidase Site-Directed Mutants

A number of site-directed mutants of ERManI have been generated to analyze the effects of altering selected residues on glycan binding and catalytic efficiency (Karaveg and Moremen, 2005; Karaveg *et al.*, 2005). Residues were selected based on their positions in the α -mannosidase structure, and mutations were generally either conservative substitutions or replacements with Ala residues. Mutagenesis was accomplished in the pPICZ α vector using the QuikChange mutagenesis kit (Stratagene) using protocols provided by the manufacturer. After the site-directed mutagenesis, the coding regions were fully sequenced to confirm that only the desired mutation was introduced into the recombinant protein product. All mutant proteins were expressed and purified under identical conditions as the wild-type enzyme, and in most instances the protein yields were similar to the wild-type enzyme. For catalytically inactive mutant enzymes, protein purification profiles were followed by immunoblots using antibodies raised to the recombinant wild-type proteins.

Enzyme Expression and Purification

The Class 1 α -mannosidases can be readily expressed in *P. pastoris*, either in shake flasks (Karaveg *et al.*, 2005) or small fermentor cultures (Tempel *et al.*, 2004). For shake flask cultures, yeast transformants were grown for 2 days in 1L BMGY (400 mg of biotin, 0.5% methanol, 1% glycerol, 1% yeast extract, 2% peptone, 0.1 M potassium phosphate [pH 6], and 1.34% yeast nitrogen base), at 30°, with agitation at 300 rpm to generate cell mass; recombinant protein production was initiated by the addition of 0.5% methanol to the culture. Additional methanol (0.5% final concentration) was then added at 12-h intervals for 3 to 5 days. Alternatively, protein production can be generated in a New Brunswick BioFlow 3000 fermentor (1- or 2.5-l vessels) in BMGY medium supplemented by a limiting feeding strategy using glycerol as a carbon source until a cell density of approximately 200 g/l is attained. The glycerol feed was then stopped, and the culture was maintained at 30° with agitation until oxygen consumption fell, indicating that the carbon source was consumed. Methanol was then pumped into the fermentor vessel and maintained at 0.5% by the use of a methanol probe (Raven Biotech, Vancouver, BC) controlling the methanol feed pump. The culture was then maintained at this methanol concentration for an additional 3 days of culture before harvesting.

Initial purification of the secreted α -mannosidase catalytic domains followed a clarification of the culture supernatant by centrifugation (6000 $\times$ g, 20 min) and filtration through a 0.4- μ m filter. For GolgiManIA (Tempel *et al.*, 2004), the resulting supernatant was dialyzed against four volumes of 10 mM sodium succinate (pH, 6.5) with two changes of buffer, followed by adjustment of the pH to 5.5 with HCl immediately before chromatography. The human ERManI (Karaveg *et al.*, 2005) culture supernatant was adjusted to pH 6.0 and

applied to the cation exchange column after 1:2 dilution with H₂O. Both enzyme preparations were loaded on SP Sepharose columns (Amersham-Pharmacia Biotech, 5 × 13 cm column), washed with column buffer, and elution was performed with a linear NaCl gradient (0 to 0.5 M) in the same buffer. For GolgiManIA purification, the column buffer was 10 mM sodium succinate (pH, 5.5); and for the ERManI purification, the column buffer was 10 mM sodium succinate (pH, 6.0), 1 mM CaCl₂. ERManI preparations were sufficiently enriched after the SP Sepharose step to proceed directly to gel filtration. GolgiManIA preparations were further purified by adjustment of the solution to 1.0 M (NH₄)₂SO₄, 0.1 M Na-HEPES (pH, 6.5), followed by loading on a Phenyl Sepharose column (Amersham-Pharmacia Biotech; 1.5 × 20 cm). The column was washed with 1.0 M (NH₄)₂SO₄ and 10 mM Na-HEPES (pH, 6.5) and eluted using a linear gradient of 1.0 to 0.5 M (NH₄)₂SO₄ in 10 mM Na-HEPES (pH, 6.5). For both enzyme preparations, fractions containing enzyme activity were pooled and concentrated using an Amicon YM10 membrane after the addition of NDSB-201 (Calbiochem) to 0.75 M. Aliquots of 5 ml of concentrated enzyme sample were applied to a Superdex 75 column (Amersham-Pharmacia Biotech, 1 × 100 cm) pre-equilibrated with 20 mM Na-MES (pH, 6.5 for mouse GolgiManIA; pH, 7.0 for human ERManI), 150 mM NaCl, 5 mM CaCl₂, and 0.25 M NDSB-201. The final purified enzyme preparations were concentrated to 30 mg/ml in the same buffer and stored at 4°. The inclusion of NDSB-201 in all of the buffers after the Phenyl Sepharose step was an important element in the purification strategies. Significant losses in the recovery of the recombinant enzymes by aggregation and precipitation were avoided by inclusion of NDSB-201 in all of the buffers.

Oligosaccharide Isolation for Enzyme Assays and Binding Studies

Man₉GlcNAc₂ glycan structures were preparatively isolated from crude soybean agglutinin (SBA) generated from soybean meal by acid extraction and (NH₄)₂SO₄ precipitation (Karaveg and Moremen, 2005; Karaveg *et al.*, 2005). Crude SBA was subsequently denatured, reduced, and carboxyami-domethylated in 8 M guanidine HCl containing 50 mM dithiothreitol and 100 mM iodoacetamide before proceeding to trypsin/elastase digestion. Glycopeptides were recovered by affinity chromatography using concanavalin A-Sepharose (ConA-Sepharose, Amersham-Pharmacia Biotech) and further purified by high performance liquid chromatography (HPLC) on a Cosmosil C18 column for surface plasmon resonance (SPR) studies (Karaveg *et al.*, 2005). Free oligosaccharides were liberated from the peptide by peptide:N-glycosidase F digestion and derivatized with pyridylamine. Man₈GlcNAc₂-PA isomers, Man₆GlcNAc₂-PA, and Man₅GlcNAc₂-PA were generated by digestion with either ERManI or GolgiManIA and isolation by reverse phase HPLC, as described (Karaveg and Moremen, 2005; Karaveg *et al.*, 2005).

α-Mannosidase Assays and Kinetic Analyses

Class I mannosidase enzyme activity can be measured by monitoring the cleavage of Man-α1,2-Man disaccharides or pyridylamine-tagged high-mannose oligosaccharides (Man₉GlcNAc₂-PA). Assays using tagged high-mannose oligosaccharides as substrates are more sensitive than the disaccharide assays, but they are more labor intensive and not as readily adapted to a high-throughput assay format. An alternative assay using a [³H]mannose-labeled Man₉GlcNAc₂ substrate followed by detection of cleavage by substrate binding to ConA has also been described (Herscovics and Jelinek-Kelly, 1987), but this requires the generation and handling of hazardous radiolabeled substrates.

Disaccharide assays for α-mannosidase activity make use of a modified glucose oxidase/horseradish peroxidase assay (Lal *et al.*, 1998). Assays were assembled in flat-bottomed 96-well microliter plates containing 10 mM potassium phosphate (pH, 6.0), 2 to 10 mM

Man α 1,2Man α -O-CH₃ (Sigma), and 5 to 15 μ l of enzyme solution (25 μ l total volume) followed by incubation at 37° for 30 min. Reactions were terminated by adding 25 μ l of 1.25 M Tris-Cl (pH, 7.6), and the amount of mannose released was detected by incubation with 250 μ l of developing solution containing glucose oxidase (55 U/ml), horseradish peroxidase (1 purpurogallin unit/ml), and o-dianisidine dihydrochloride (70 μ g/ml) for 3 h at 37°. The final color intensity was determined by measuring the absorbance at 450 nm. Free mannose was used as a standard. One unit of mannosidase activity is defined as the amount of enzyme that releases 1 nmole of mannose in 1 minute at 37°.

Assays using oligosaccharide substrates (Lal *et al.*, 1998) contained 100 pmol Man₉GlcNAc₂-PA, 50 mM MES (pH, 7), 150 mM NaCl, and 5 mM CaCl₂ (20 μ l total volume), and reactions were performed at 37° for varied periods of time, depending on the concentration of the enzyme. Reactions were stopped by the addition of 20 μ l 1.25 M Tris-HCl (pH, 7.6), followed by separation of the digested products on a Hypersil APS- 2 NH₂ column (4.6 $\times$ 250 mm, Alltech), using a gradient from 90% buffer A (80% acetonitrile : 20% 50 mM Na phosphate; pH, 4) to 35% Buffer B (100 mM Na phosphate; pH, 4) run over 50 min at flow rate of 1 ml/min. Elution of the PA-tagged oligosaccharide was monitored by an in-line fluorescence detector (excitation wavelength, 320 nm; emission wavelength, 400 nm), with identification of the cleavage products based on a comparison to the elution positions of Man₉₋₅GlcNAc₂-PA standards. One unit of enzyme activity generated 1 μ mol/min of Man₈GlcNAc₂ from Man₉ GlcNAc₂ at 37°. An alternative solvent protocol provided a shorter HPLC separation time. Using 40% acetonitrile in 60 mM Na phosphate buffer (pH, 4) with an isocratic flow of 1.0 ml/min, Man₉₋₅GlcNAc₂-PA could be resolved in less than 15 min. Variations in the flow rate (0.7 to 1.4 ml/min) or an increase in the percentage of acetonitrile by 5% to 15% can be used to shift the elution positions of the high-mannose glycans for optimal resolution.

For kinetic analysis (Karaveg and Moremen, 2005; Karaveg *et al.*, 2005), initial rates (v) for the enzyme reactions were determined at various substrate concentrations ranging from 10 to 300 μ M. Catalytic coefficient (k_{cat}), and Michaelis constant (K_m) values were determined by fitting initial rates to a Michaelis-Menten function by nonlinear regression analysis using SigmaPlot (Jandel Scientific, San Rafael, CA). k_{cat}/K_m values could also be derived from reciprocal plots of v and $[S]$. For kinetic pH rate analysis (Karaveg *et al.*, 2005), values of k_{cat} and K_m were determined from initial rates of enzyme reactions in the pH range from 4 to 10 using a 4 $\times$ universal buffer (80 mM succinic acid, 80 mM MES, 80 mM HEPBS, 80 mM HEPES, and 80 mM CHES adjusted to pH with 5 M NaOH) in place of the MES assay buffer described previously. Plots of $\log(k_{cat}/K_m)$ versus pH could then be used to estimate the pK_a values for the acidic and basic limbs of the pH curves for the wild-type and mutant enzymes. In studies on the temperature dependence of catalysis (Karaveg and Moremen, 2005), values for k_{cat} were obtained between 5° and 40° at 5° intervals and were used to calculate activation energies (E_a) from the slopes ($-E_a/R$) of Arrhenius plots ($\ln(k_{cat})$ as a function of $1/T$). The thermodynamic activation parameters can then be calculated from the plots as previously described (Karaveg and Moremen, 2005).

Glycan Binding Studies by SPR

The combination of mutagenesis and substrate binding studies by SPR provided a useful set of tools for investigating the determinants of binding kinetics and glycan affinity for the recombinant α -mannosidases (Karaveg and Moremen, 2005; Karaveg *et al.*, 2005). Among the various strategies for binding studies by SPR, we have chosen to immobilize the enzymes to the sensor chip surface and screen for glycan binding as the mobile phase analyte (Fig. 2).

SPR analyses were conducted using a Biacore 3000 apparatus (Biacore AB), with recombinant enzymes immobilized on the chip surfaces at 25° by amine coupling. The flow cells were activated by injecting a mixture of 50 mM *N*-hydroxysuccinimide and 200 mM 1-ethyl-3-(dimethylaminopropyl) carbodiimide over the CM5 sensor chip surface for 7 min at 5 µl/min. The recombinant proteins prepared in 10 mM sodium succinic acid (pH, 6.0) were passed through a 0.2-µm filter and diluted in the same buffer to obtain a concentration of 5 µg/ml before injection onto the activated surface. The desired immobilization level can be achieved by specific contact time with the chip surface. The immobilization efficiency for ERMAnI was about 2500 RU/min at a flow rate of 5 µl/min in HPB-EP buffer (10 mM HEPES; pH, 7.4; 150 mM NaCl; 3.4 mM EDTA; 0.01% polysorbate P20) at 25°. The remaining reactive groups were blocked by injection of 1 M ethanolamine-HCl at pH 8.5 for 7 min at 5 µl/min. Mock derivatized flow cells served as reference surfaces. Binding analyses were generally performed at 10° with a continuous flow (30 µl/min) of running buffer (10 mM MES; pH, 7.0, 300 mM NaCl; and 5 mM CaCl₂) except in temperature-dependent interaction studies, which have been performed between 5° and 35° at 5° intervals, consecutively, in an automated method. Analytes were prepared in running buffer by twofold serial dilution to obtain appropriate concentration ranges. A concentration series of Man₉GlcNAc₂-glycopeptide (0.4 to 400 µM) was analyzed over a low-density immobilization surface of recombinant protein (3000 RU), whereas disaccharides (16 to 1000 µM) and small molecule inhibitors, such as 1-deoxymannojirimycin (dMNJ) (2 to 1000 µM) and kifunensine (Kif), were analyzed over a high-density immobilization surface of recombinant protein (10,000 RU). Samples were injected for 1 min, followed by at least 3 min of dissociation phase. In most cases, the baseline returned to the original response level in 5 min without a further regeneration of the chip surface, except when Kif was used as the analyte (see Fig. 2). This latter inhibitor exhibits tight-binding kinetics and does not dissociate from the chip surface even with extensive washing. SPR data for each concentration of analyte were collected in duplicate and globally fit to a 1:1 Langmuir binding algorithm model to calculate the on-rate (k_a), the off-rate (k_d), and the equilibrium dissociation constant ($k_d/k_a = K_D$) using BiaEvaluation version 3.1 software supplied with the instrument. Alternatively, the maximal equilibrium sensorgram values were used to plot a saturation binding curve and calculate values for the equilibrium dissociation constant (K_D) directly. K_D values measured at different temperatures can also be used to calculate thermodynamic parameters (ΔG , ΔH , $T\Delta S$) of binding (Karaveg and Moremen, 2005) using the van't Hoff equation (Day *et al.*, 2002) to plot the linear relationship of $y = \ln(K_D)$ versus $x = 1/T$, which gives a slope of $\Delta H/R$ and an intercept of $-\Delta S/R$. The effects of temperature on the individual association rates (k_a) and dissociation rates (k_d) can also be independently determined using the Eyring equation (Day *et al.*, 2002), which allows thermodynamic parameters to be determined from measured k_a and k_d values at different temperatures by a linear relationship of $y = R\ln(hk_a/k_B T)$ or $y = R\ln(hk_d/k_B T)$ versus $x = 1/T$, where the slope and the intercept of the Eyring plots are $\Delta H^\ddagger$ and $\Delta S^\ddagger$, respectively (Karaveg and Moremen, 2005).

Summary

The biochemical characterization of Class 1 α -mannosidases has made use of a combination of approaches including recombinant expression of wild-type and mutant enzymes, purification by classical chromatography approaches, analyses of enzyme kinetics, and binding affinity and kinetic measurements using SPR (Karaveg and Moremen, 2005; Karaveg *et al.*, 2005). In the case of human ERMAnI, these approaches have been complemented by structural determination of co-complexes between the enzyme and small molecule glycone mimics, uncleavable disaccharide pseudo-substrates, and large glycan enzymatic products (Karaveg *et al.*, 2005; Tempel *et al.*, 2004; Vallee *et al.*, 2000a,b). The result of these studies was a detailed map of the energetic contributions to substrate

recognition, binding, and catalysis (Fig. 3) (Karaveg and Moremen, 2005) that will establish a framework for studying the differences in recognition between the different Class 1 α -mannosidase subfamilies. Since these proteins play distinct roles in glycoprotein maturation and ER quality control, further study should lead to a greater understanding of how these common sequences and structural folds can accomplish their unique biological functions. The strategies used for the biochemical characterization of the α -mannosidases can also be applied to the study of many other families of enzymes that play critical cellular functions.

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Fig. 1.

Diagram of the early *in vivo* N-glycan processing steps of $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ -Asn to $\text{Man}_5\text{GlcNAc}_2$ -Asn. The processing steps catalyzed by glucosidases I and II remove the three glucose residues. Trimming by ERManI generates the Man8 isomer B, and trimming by the GolgiManI subfamily of enzymes generates $\text{Man}_5\text{GlcNAc}_2$. The GlcNAc residues are indicated by black shaded hexagons. Mannose residues are gray hexagons. Glc residues are white hexagons.

Fig. 2.

Examples of SPR binding of Man₉GlcNAc₂, dMNJ, or Kif ligands to wild-type and mutant ERMAnI. Wild-type (A, B, and F) or mutant (C, D, and E) ERMAnI forms were immobilized on the SPR chip surface and various concentrations of Man₉GlcNAc₂ (left panels), dMNJ (B and D), or Kif (F) ligands were tested for binding. Representative SPR sensorgrams in the ligand concentration series are shown. If the on- and off-rates (k_a and k_d , respectively) were sufficiently slow (B and E), curve fitting of the sensorgrams was performed to determine values for the equilibrium dissociation constants ($K_D = k_d/k_a$). Where the kinetics for ligand binding were too rapid, equilibrium sensorgram values were used to plot a saturation curve (insets in A and C) and calculate values for K_D . In the F659A mutant, binding of

Man₉GlcNAc₂ was minimally affected (C), whereas dMNJ binding was completely abolished, as indicated by the absence of a deflection in the SPR sensorgram trace (D). For panel F, the tight-binding kinetics of Kif resulted in no appreciable dissociation from the chip surface. Reproduced in part from Karaveg and Moremen (2005), with permission of American Society for Biochemistry and Molecular Biology via Copyright Clearance Center.

Fig. 3.

Summary of the enzyme kinetics and binding studies for key protein and glycan residues in the ERManI active site. The catalytic and binding components in the ERManI active site are shown, highlighting the protein residues interacting with the -1, +1, and +2 subsite glycan residues. Fold differences for kinetic parameters (values for k_{cat} and K_m) and SPR binding parameters (K_D values for binding to Man₉GlcNAc₂ and dMNJ) for the mutant enzymes relative to values for wild-type ERManI are indicated adjacent to each respective amino acid. In addition, the respective proposed functions for each residue based on the binding and kinetic analyses are indicated adjacent to each amino acid. Energetic contributions to binding within the -1 and +1 subsites are also indicated by the dotted lines with shading. Reproduced from Karaveg and Moremen (2005), with permission from American Society for Biochemistry and Molecular Biology via Copyright Clearance Center.

TABLE I

Constructs for Expression of Class 1 α -Mannosidases in *Pichia Pastoris*

Class 1 α -mannosidase ^a	<i>Pichia</i> host strain	Expression vector	Residues of coding region expressed	Amino acids in full-length coding region	Predicted <i>N</i> -glycan acceptor sites
Yeast ERManI	GS115, KM71	pHILS-1	34–549	549	3
Human ERManI	X33	pPICZ- α	226–699	699	0
Mouse GolgiManIA	GS115	pHILS-1	66–655	655	1
Human EDEM2	KM71H	pPICZ- α	18–492	578	4

^aInformation on the constructs and strains for the expression data of yeast ERManI (Lipari and Herscovics, 1996), human ERManI (Karaveg *et al.*, 2005), mouse GolgiManIA (Lal *et al.*, 1998), and human EDEM2 (unpublished data) were obtained from the respective sources.