Structural Basis for Catalysis and Inhibition of N-Glycan Processing Class I α1,2-Mannosidases

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Endoplasmic reticulum (ER) class I α1,2-mannosidase (also known as ER α-mannosidase I) is a critical enzyme in the maturation of N-linked oligosaccharides and ER-associated degradation. Trimming of a single mannose residue acts as a signal to target misfolded glycoproteins for degradation by the proteasome. Crystal structures of the catalytic domain of human ER class I α1,2-mannosidase have been determined both in the presence and absence of the potent inhibitors kifunensine and 1-deoxymannojirimycin. Both inhibitors bind to the protein at the bottom of the active-site cavity, with the essential calcium ion coordinating the O-2 hydroxyls and stabilizing the six-membered rings of both inhibitors in a C4 conformation. This is the first direct evidence of the role of the calcium ion. The lack of major conformational changes upon inhibitor binding and structural comparisons with the yeast α1,2-mannosidase enzyme-product complex suggest that this class of inverting enzymes has a novel catalytic mechanism. The structures also provide insight into the specificity of this class of enzymes and provide a blueprint for the future design of novel inhibitors that prevent degradation of misfolded proteins in genetic diseases.

Endoplasmic reticulum (ER) class I α1,2-mannosidase (also

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The atomic coordinates and structure factors (code 1FM1, 1F02, and 1FO3) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org).** To whom correspondence should be addressed: Structural Biology and Biochemistry, Research Inst., Hospital for Sick Children, 555 University Ave., Toronto, Ontario M5G 1X8, Canada. Tel.: 416-813-5378; Fax: 416-813-5022; E-mail: howell@sickkids.on.ca.

1 The abbreviations used are: ER, endoplasmic reticulum; HM, human ER class I α1,2-mannosidase; KIF, kifunensine; dMNJ, 1-deoxymannojirimycin; NDSB, non-detergent sulfobetaine; MES, 4-morpholineethanesulfonic acid; Wat, water.

Known as ER α-mannosidase I and Man₉GlcNAc₂-specific processing α-mannosidase, EC 3.2.1.113) is a key enzyme in the maturation of N-linked oligosaccharides in mammalian cells (for reviews, see Refs. 1–5). N-Glycan formation begins with the transfer of a preformed oligosaccharide precursor, Glc₃Man₉GlcNAc₂, to the nascent polypeptide chain. α-Glucosidases and α-mannosidases in the ER trim this oligosaccharide precursor to primarily Man₉GlcNAc₂, while subsequent transport of the glycoprotein into the Golgi apparatus allows further trimming of the remaining α1,2-linked mannose residues. The resulting Man₉GlcNAc₂ oligosaccharide is the substrate for GlcNAc-transferase I, the first enzyme in a cascade that ultimately results in the formation of complex and hybrid oligosaccharide structures (6). ER class I α1,2-mannosidase also plays a key role in the degradation of misfolded glycoproteins (7–11). The trimming of a single mannose from Man₉GlcNAc₂ creates a signal that targets misfolded glycoproteins for translocation out of the ER and degradation by the proteasome (for review, see Ref. 12).

Human ER class I α1,2-mannosidase (HM) was recently cloned (13, 14) and found to share significant sequence similarity with the ER α1,2-mannosidase from Saccharomyces cerevisiae (15) and to mammalian Golgi class I α1,2-mannosidases. These enzymes, members of Family 47 of the glyco- sylhydrolases (16), are type II membrane proteins with an N-terminal cytoplasmic domain, a single transmembrane helix, and a large luminal C-terminal catalytic domain. Class I α1,2-mannosidases are calcium-dependent enzymes (1–5, 17) that cleave α1,2-linked mannose units with inversion of anomeric configuration (18). The ER-resident enzyme removes a single mannose from the middle arm of Man₉GlcNAc₂ to form Man₈GlcNAc₂ isomer B (13, 19, 20), whereas the Golgi enzymes remove all four α1,2-linked mannose units (21–28).

The structure of the catalytic domain of the ER α1,2-mannosidase from S. cerevisiae has recently been determined by x-ray crystallography (29). The protein is an (αα)₇-helix barrel with one side of the barrel plugged by a β-hairpin. The nine highly conserved acidic residues and the calcium ion, all of which are essential for catalytic activity (30), are located at the top of this β-hairpin in the center of the barrel, at the bottom of a 15-Å deep cavity. In the structure, an N-glycan from one molecule interacts with a symmetry-related molecule in what is believed to be an enzyme-product complex. This interaction helped to identify the active site and residues involved in oligosaccharide binding. Examination of the protein-carbohydrate interaction revealed an arginine residue (Arg273 in yeast α1,2-mannosidase) that interacts with three mannose residues and one N-acetylglucosamine residue of the N-glycan. This interaction has
been shown to be responsible, in part, for the specificity of yeast ER class I α1,2-mannosidase (31). In mammalian Golgi α1,2-mannosidases that trim Man \( _9 \)GlcNAc \( _2 \) to Man \( _5 \)GlcNAc \( _2 \), this arginine residue is typically a leucine. Replacement of Arg \( ^{273} \) with leucine in yeast ER class I α1,2-mannosidase results in an enzyme that is capable of cleaving all four α1,2-linked mannose residues rather than just the single terminal residue of the middle arm of Man \( _9 \)GlcNAc \( _2 \).

Although the observed protein-carbohydrate interactions in yeast ER class I α1,2-mannosidase have provided a wealth of information regarding substrate specificity, the N-glycan lacks the middle-arm terminal mannose residue that would be specifically cleaved during the enzymatic reaction. This prevented unambiguous identification of the residues involved in catalysis and provided no evidence for the role of the calcium ion. We present here the first structures of a class I α1,2-mannosidase in complex with the potent inhibitors kifunsenine (KIF) (32) and 1-deoxynojirymicin (dMNJ) (33). The structural results provide clear evidence for the role of calcium in substrate stabilization and suggest that this class of inverting enzymes has a novel catalytic mechanism. This work also provides insight into the specificity of these enzymes for α1,2-linked mannose residues and the inhibitors kifunsenine and 1-deoxynojirymicin.

The importance of ER class I α1,2-mannosidases in ER-associated degradation of misfolded glycoproteins has been demonstrated in both yeast and mammalian cells. In yeast, it has been shown that a misfolded mutant of carboxypeptidase \( \gamma \) is rapidly degraded in wild-type cells, whereas it is stabilized in the mns1 mutant lacking the ER processing \( \alpha \)-1,2-mannosidase (8, 11). In mammalian cells, treatment with the α1,2-mannosidase inhibitors 1-deoxynojirymicin and kifunsenine has been shown to block the degradation of the T cell receptor subunit CD3-\( \gamma \) (34), tyrosinase (35), α2-plasmin inhibitor (36), and a misfolded variant form of \( \alpha_1 \)-antitrypsin (10). In the case of \( \alpha_1 \)-antitrypsin, increased secretion of \( \alpha_1 \)-antitrypsin was also observed (37). Since the aggregation of misfolded \( \alpha_1 \)-antitrypsin in the ER leads to emphysema, understanding the structural basis of inhibition of class I α1,2-mannosidase is therefore the first step toward the structure-based design of novel therapeutic agents for this and other genetic diseases characterized by rapid degradation of misfolded glycoproteins.

EXPERIMENTAL PROCEDURES

Expression and Purification—The cloning and characterization of the soluble catalytic domain of human ER class I α1,2-mannosidase as a protein A fusion (pPROTA-ERManI) were described previously (13). The portion of the cDNA encoding the catalytic domain (amino acids 172–689) was excised from the pPROTA expression vector by digestion with EcoRI and ligated into the EcoRI site of the Pichia expression vector pPICZaA (Invitrogen, La Jolla, CA). The final construct in the expression vector (10 \( \mu \)g) was linearized by digestion with HindIII and transformed into Pichia pastoris host strain X-33 by the lithium chloride transformation method as described in the Pichia expression manual (Invitrogen). Transformants were selected on yeast extract, peptone, dextrose (YPD)/Zeocin (100 \( \mu \)g/ml) plates and screened for expression of the recombinant human ER α1,2-mannosidase enzyme activity as described below following methanol induction in small-scale liquid cultures (24, 38–41). The Pichia transformant expressing the highest level of the soluble enzyme activity was used to produce the recombinant enzyme in large shaker flask and fermentor cultures. After optimizing the expression and purification of human ER α1,2-mannosidase from 1-liter shaker flask cultures, enzyme expression was scaled-up by growth of the culture in a 10 liter fermentor (New Brunswick Scientific, NJ) at the Fermentation Research Facility of the University of Georgia. The fermentor, containing 100 liters of BMGY medium (400 \( \mu \)g/ml of biotin, 0.5% methanol, 1% glycerol, 1% yeast extract, 2% peptone, 0.1 M potassium phosphate (pH 7), and 1.34% yeast nitrogen base), was inoculated with a 5-liter overnight culture of the human ER α1,2-mannosidase Pichia transformant in BMGY medium. The culture was maintained at 30 °C with agitation at 180 rpm and an air flow of 100 liters/min for 48 h until the glycerol in the culture was consumed. Human ER α1,2-mannosidase enzyme expression was then induced by the daily addition of methanol (50% (v/v) stock solution) to a final concentration of 0.5%. After 5 days of induction, the medium was harvested using a Sharples Model AS-16P continuous centrifuge at 15,000 rpm for 5 h, followed by ultrafiltration using a 30% membrane. Attempts to concentrate the culture medium by ultrafiltration resulted in >50% loss of enzyme activity and the appearance of human ER α1,2-mannosidase as a precipitate on the ultrafiltration filter. As a result, the clarified culture medium was not concentrated, but was applied directly in 20-liter batches to an SP-Sepharose column (5 × 13 cm; Pharmacia Biotech) at a flow rate of 20 ml/min. The column was washed with 500 ml of column buffer containing 10 mM sodium succinate (pH 6.0) and 1 mM CaCl\(_2\) and eluted with a one-liter linear gradient of 0–0.5 M NaCl in the same column buffer at a flow rate of 6 ml/min. The protein eluted at a sharp peak at ~400 mM NaCl. The fractions containing enzyme were pooled and concentrated by ultrafiltration through a YM-10 membrane (Amicon, Inc., Beverly, MA). The solubility of the concentrated protein was maintained by the addition of NDSB201 (Calbiochem) (42–44) to 1.0 M. After concentration, an EDTA-free protease inhibitor mixture (Roche Molecular Biochemicals, Mannheim, Germany) was added to prevent protease degradation. The concentrated enzyme preparation, in 7–ml batches, was further purified by loading onto a Superdex 75 gel filtration column (1.6 × 65 cm; Amersham Pharmacia Biotech) pre-equilibrated with 10 mM sodium succinate (pH 7.0), 150 mM NaCl, 5 mM CaCl\(_2\), and 0.25 mM NDSB201. Fractions containing human ER α1,2-mannosidase were pooled and concentrated to 1.8 mg/ml using a YM-10 membrane. The concentrated enzyme was stored at 4 °C prior to use in crystallization trials. An aliquot of the purified enzyme (50 \( \mu \)g) was subjected to SDS-polyacrylamide gel electrophoresis (45) and transferred to a polyvinylidene difluoride membrane to determine the N-terminal sequence as described previously (24). The protein (157 mg) was purified with a 16.7% yield from 100 liters of fermentor medium.

Solubility of Purified Recombinant Enzyme in the Presence of Non-detergent Sulfobetaines (NDSBs)—During purification, the recombinant protein was found to rapidly precipitate when stored at 4 °C, even at low protein concentrations (<0.1 mg/ml). A series of solubility tests were therefore performed to investigate the effect of salts, detergents, glycerol, and NDSBs (43, 44) on the solubility of the enzyme (data not shown). To test conditions for maintaining soluble enzyme during the crystallization trials, the purified enzyme (0.14 mg/ml) was concentrated in the presence of either 0.75 or 0.25 mM NDSB256, NDSB201, or NDSB195 (Calbiochem) (43, 44) or 1% glycerol using a Centricron-10 concentrator (Amicon, Inc., Beverly, MA). The concentrated enzyme was stored at 4 °C for 5 days. Aliquots were removed daily, and after centrifugation at 16,000 × g for 1 min, the protein concentration of the supernatant was determined as described below. Glycerol and nonionic detergents were found to have a minimal effect on the solubility of the enzyme, with only 20–30% of the protein remaining soluble after concentration and 10–20% remaining soluble after 5 days. In contrast, samples containing the NDSB compounds resulted in ~65–81% of the enzyme remaining soluble after concentration and 25–40% remaining soluble after 5 days (data not shown). The most effective solubilizing agent was NDSB201. In NDSB201, 81% of the protein remained soluble after concentration, and 30% remained soluble after 5 days at 4 °C. As a result of the solubility studies, the pooled enzyme preparation after the SP-Sepharose step was concentrated in the presence of 1.0 mM NDSB201 prior to its application to the Superdex 75 column (as described above), and the gel filtration column was run with a buffer containing 0.25 mM NDSB201.

Enzyme and Protein Assays—Human ER α1,2-mannosidase enzyme activity was determined by the addition of the enzyme sample to a final reaction volume of 40 \( \mu \)l containing 20 mM MES (pH 7.0), 150 mM NaCl, 5 mM CaCl\(_2\) and pyridylamine-tagged (PA) Man\(_9\)GlcNAc\(_2\), as an oligosaccharide substrate. The assays were incubated for 10–20 min at 37 °C, and the reactions were stopped by heating to 100 °C for 5 min. The human ER α1,2-mannosidase product, Man\(_9\)GlcNAc\(_2\)-PA, was released from Man\(_9\)GlcNAc\(_2\)-PA on a Hypersil APS-2 NH\(_2\) high-pressure liquid chromatography column (13, 38). One unit of enzyme activity is defined as the amount of enzyme that releases 1 pmol of Man\(_9\)GlcNAc\(_2\)-PA from Man\(_9\)GlcNAc\(_2\) in 1 min at 37 °C and pH 7.0. Protein concentration was determined using the BCA protein assay reagent (Pierce) as described by the manufacturer.

Crystallization and Initial Data Collection—Crystals of human ER α1,2-mannosidase were grown using the hanging-drop vapor diffusion method from equal volumes of protein solution (10 mg/ml in 20 mM MES (pH 7), 150 mM NaCl, 5 mM CaCl\(_2\), and 0.25 mM NDSB201) and precipi-
tating solution (1.6–1.7 M (NH₄)₂SO₄) suspended over a 1-mL reservoir containing the same precipitating solution. Rod-shaped crystals grew within ~5 days to a maximum size of 0.4 × 0.2 × 0.2 mm. The crystals are trigonal and belong to space group P3₁2₁ with unit dimensions of a = b = 95.8 Å, c = 136.8 Å, and γ = 120°. One molecule is present in the asymmetric unit. The complexes of human ER α,2-mannosidase with 1-deoxymannojirimycin and kifunensine were prepared by controlled crystallization. The conditions described above for the uncomplexed protein were used with the inhibitor added to the precipitating solution. The concentration of inhibitor (0.5–25 mM) was screened to determine which concentration of inhibitor produced the best crystals. X-ray diffraction data were collected on crystals containing 0.5 mM kifunensine or 6.5 mM 1-deoxymannojirimycin. The protein-inhibitor crystals have the same space group and unit cell dimensions as the native uncomplexed crystals (see Table I). An initial set of data for the uncomplexed protein was collected from a single crystal at room temperature using CuKα radiation (Rigaku RotaFlex RU200 rotating anode generator) on a Mar Research image plate detector (345-mm diameter). The crystal diffracted to a minimum d-spacing of 2.8 Å. High-resolution data for the native and inhibitor complexed crystals were collected at Beamline X8-C at the National Synchrotron Light Source (Brookhaven National Laboratory, Upton, NY) using a Quantum4 CCD detector and flash-flooded with a 20% (v/v) glycerol/artificial mother liquor solution.

**Structure Determination and Refinement**—The uncomplexed human ER α,2-mannosidase structure was solved by molecular replacement using the AMoRe program package (47) and the 2.8 Å room temperature data set (see Table I). The coordinates of yeast α,2-mannosidase (Protein Data Bank code 1DL2) (29) were used as the search model with the following modifications: amino acid residues not conserved between the human and yeast sequences were truncated to alanine, and loops not present in the human enzyme were omitted. Rotation functions were calculated over a resolution range of 15 to 4 Å with a Patterson radius of 20 Å. The initial molecular replacement solution gave Rcryst = 46.4% and a correlation coefficient of 35.9%. The structure was refined using the CNS program suite (48). A maximum likelihood target (49) with a flat bulk solvent correction and no low resolution or cutoff applied to the data was used in the refinement protocol. Ten percent of the structure factors were randomly selected, excluded from the refinement, and used to compute Rfree (50). Refinement of the model using the simulated annealing slow-cooling protocol (51, 52) was alternated with manual inspection and rebuilding of the model using TURBO-FRODO (53). After 16 cycles of refinement and manual rebuilding, 455 residues of the protein had been modeled. The structure comprises residues 241–388 and 391–697 with overall dimensions of approximately 22.9 × 136.8 × 178.5 Å. The crystals grow with a flat bulk solvent correction and no low resolution or cutoğ applied to the data and were used in the refinement protocol. Ten percent of the structure factors were randomly selected, excluded from the refinement, and used to compute Rfree (50). Refinement of the model using the simulated annealing slow-cooling protocol (51, 52) was alternated with manual inspection and rebuilding of the model using TURBO-FRODO (53). After 16 cycles of refinement and manual rebuilding, 455 residues of the protein had been modeled. The structure comprises residues 241–388 and 391–697. Residues 226–240, 389–390, and 698–699 could not be located owing to weak electron density in these regions. This model was subsequently used to refine the high-resolution native human ER α,2-mannosidase (1.9 Å, HM-KIF22), and HM-MNJ12, and HM-MNJ12 and HM-MNJ12 structures at resolutions of 1.75Å and 2.4Å, respectively. In each case, difference electron density clearly defined the position and orientation of the inhibitor molecule (Fig. 1, a and b). The HM-KIF and HM-MNJ1 structures have been refined to Rcryst = 21.9% and Rfree = 24.1% and Rcryst = 19.0% and Rfree = 23.9%, respectively (Table I).

**Overall Structure of the Molecule**—The core of the human ER α,2-mannosidase structure is an (αα)-barrel composed of 14 consecutive helices alternating from outside to inside the barrel (Fig. 2, A–C). The barrel has an approximate internal 7-fold symmetry. This arrangement of helices results in a topology of seven parallel inner helices (α2, α4, α6, α8, α10, α12, and α14) and seven parallel outer helices (α1, α3, α5, α7, α9, α11, and α13), which are oriented concentrically toward the inner helices and anti-parallel to them. The structure is stabilized by a unique disulfide bond, Cys857–Cys858, which forms a bridge between helices 31αb and 11α (Fig. 2C). This disulfide bridge occurs between residues conserved in all members of the class I α,2-mannosidase family and has been shown to be essential for activity (74).

The two ends of the (αα)-barrel are structurally distinct (Fig. 2B). On one side, the short connection (SC side), the pairs of inner and outer helices are connected by short loops of up to four residues. The opposite side, the long connection (LC side), consists of a complex array of β-strands. The active site is on the long connection side of the protein. The C-terminus of the protein consists of a β-hairpin protruding back into the center of the inner barrel from the short connection side. This β-hairpin plugs the inner barrel and prevents the core of the protein from being an open channel. The β-hairpin, the inner helices, and the β-sheets on the long connection side of the barrel form a cavity ~15 Å deep, with an upper diameter of ~25 Å decreasing to ~10 Å at the top of the β-hairpin.

**Calcium Binding in Human α,2-Mannosidase**—Calcium is essential for the activity of class I α-mannosidases (1–5, 17) and has been shown to protect the yeast enzyme against thermal denaturation (30). In the human α,2-mannosidase structure, the calcium ion binds to the carbonyl oxygen and Oγ atoms of Thr688 located at the top of the β-hairpin and to four water molecules, which are, in turn, hydrogen-bonded to one of the carbonate groups of Glu647, Glu699, Glu602, and Glu663. Two additional water molecules complete the 8-fold pentagonal bipy-
FIG. 1. Initial $F_o - F_c$ map for HM-KIF (a) and HM-dMNJ (b) complexes. The maps were calculated in the absence of the inhibitor or calcium ion. The maps are contoured at +4σ (thin lines) and +20σ (thick lines) for HM-KIF and at +3.5σ (thin lines) and +15σ (thick lines) for HM-dMNJ. The 1-deoxymannojirimycin in b is viewed at −90° to the orientation of kifunensine. Please note that to facilitate the comparison of different inhibitors and the discussion of the catalytic mechanism, kifunensine has been numbered so that its six-membered ring has similar atom numbering to 1-deoxymannojirimycin and mannose. (This is not the standard IUPAC nomenclature.)
The calcium ion (Ca) is represented as a dark blue sphere. SC side, short connection. Also shown is a topological two-dimensional representation of the human α,2-mannosidase structure (C). The figure is oriented as described for A. α-Helices are represented as circles, and β-strands as shown as arrows. For simplicity, helices α6a and α6b and helices α14a and α14b are represented as single helices. The essential disulfide bridge at Cys^{527}–Cys^{556} (SI) is also shown. The color scheme for the α-helices is conserved in all three panels. The β-hairpin that plugs the barrel is pink, and the 3_{10} helices are black. The dashed line represents the discontinuity in the model at residues 389–390. The secondary structure of the protein (see below) was assigned with the use of the program PROMOTIF (69) and are as follows: α1, residues 248–267; β1, 273–275; β2, 280–282; α2, 289–300; α3, 304–317; β3, 325–327; α4, 328–346; α5, 349–362; β3, 363–366; β4, 376–378; β5, 384–385; β6, 393–394; α6a, 395–399; α6b, 402–412; α7, 416–429; β7, 442–443; β8, 450–451; β9, 455–456; α8, 463–475; α9, 481–497; β10, 499–501; β11, 508–510; β12, 512–514; β13, 517–519; β14, 521–523; β3, β2, 524–527; α10, 528–536; α11, 543–561; β15, 571–574; β16, 585–586; 3_{10}, 589–591; α12, 599–611; α13, 615–630; α14a, 659–662; α14b, 664–671; β17, 684–686; and β18, 692–694. N and C indicate the N and C termini of the molecule. The figure was prepared with MOLSCRIPT (70) and RASTER3D (71).
Fig. 3. Binding of 1-deoxymannojirimycin and kifunensine to human ER class I α1,2-mannosidase. A, location of kifunensine in the center of the (αα)-barrel. 1-Deoxymannojirimycin superimposes with the six-membered ring of kifunensine, but for clarity, it is not represented in this panel. The color scheme is the same as described in the legend to Fig. 2. B and C, schematic representation of the interactions between human α1,2-mannosidase and kifunensine and 1-deoxymannojirimycin, respectively. Short and long dashed lines represent hydrogen bond interactions and van der Waals contacts, respectively. For simplicity, only hydrogen bonds between the protein, water, and inhibitor molecules are represented. Water–water hydrogen bonds are not represented. D, surface representation of the catalytic cavity of human α1,2-mannosidase in the vicinity of the kifunensine-binding site. The surface is colored according to its electrostatic potential. Kifunensine is shown in stick representation. The contour level is at ±20 kT. A was prepared using MOLSCRIPT (70), and D was prepared with GRASP (72).
oxymannojirimycin and kifunensine hydrogen bond directly to Arg597, Glu399, Glu463, and Glu680 and via water molecules to Glu330, Glu502, Glu467, and Asp463 (Fig. 3, B and C). These acidic residues define the bottom of the active-site cavity and have been shown to be important for catalysis (30). The O-6' hydroxyl group of the inhibitor is completely buried in a small side recess of the active-site cavity (Fig. 3D). The O-6' hydroxyl forms a short hydrogen bond with O-e1 of Glu599 (2.5 Å) and a longer one with N-η1 of Arg597 (2.9 Å). In the case of kifunensine, additional interactions with the protein are made with atoms in the fused five-membered ring. The O-7 carbonyl and N-9 hydrogen bond directly to N-η1 of Arg597 and O-82 of Asp463, respectively. Additional hydrogen bond interactions are made via water molecules. N-9 interacts via water with O-γ of Ser464, the O-8 carbonyl with the amide nitrogen of Arg461, and the O-9 carbonyl with N-η2 of Arg597 (Fig. 3B). Most of the contacts between the protein and inhibitor molecules are electrostatic interactions. The only van der Waals interactions found are between inhibitor atoms C-4, C-5, and C-6 and Phe659 and C-7 and C-8 in kifunensine and Leu525 (Fig. 3, B and C).

The protein does not undergo any large global conformation changes upon inhibitor binding. A superposition of all C-α atoms of human α1,2-mannosidase and the HM-KIF or HM-dMNJ complex yielded a root mean square deviation of 0.25 Å for both structures. Although small variations in some side chains do occur, the largest conformational change seen upon inhibitor binding is a movement of ~1.7 Å in the side chain of Arg597. In addition to the interactions of Arg597 with the inhibitor (Fig. 3, B and C), N-η2 and N-ε of Arg597 now hydrogen bond with O-γ1 and O-γ2 of Glu570, respectively. The similarities between all three structures extend beyond the protein to the solvent. The solvent structure is essentially identical in all three protein structures, with the exception of the water molecules that are displaced from the active site upon inhibitor binding. In the HM-KIF and HM-dMNJ structures, each of the four hydroxyl oxygen atoms of the inhibitor replace a water molecule. An additional water molecule is displaced in the HM-KIF structure by N-9. The active-site cavity therefore appears to be preformed in the human α1,2-mannosidase structure prior to inhibitor/substrate binding. Only one residue, Arg597, undergoes a significant conformational change upon inhibitor binding, and ordered water molecules form hydrogen bonds with the protein in a pattern that closely mimics the hydrogen bond network found in the presence of the inhibitor. This is comparable to the active sites of other saccharide-binding proteins where ordered water molecules have been observed to mimic the positions of the inhibitor/substrate hydroxyl atoms (61).

Conformation of Kifunensine and 1-Deoxymannojirimycin—The electron density of kifunensine and 1-deoxymannojirimycin reveals that the six-membered rings of both inhibitors have a non-standard \( ^1 \)C\_4 conformation when bound at the active site of the protein. This “all-axial” conformation of kifunensine’s six-membered ring is probably the consequence of the fused five-membered ring. The energy-minimized structure of kifunensine built using the program SYBYL fit the initial 1.7 Å \( \sigma_z \)-weighted \( F_e - F_c \) difference electron density maps without any conformational rearrangement (Fig. 1a) or ambiguity in the ring pucker. In contrast, the energy-minimized 1-deoxymannojirimycin molecule, built in the \( ^4 \)C\_4 conformation, required deformation to the \( ^1 \)C\_4 conformation in order for the molecule to fit the electron density (Fig. 1b). The conformation of the six-membered ring of 1-deoxymannojirimycin is almost certainly defined by its interactions with the protein (see above). The lack of conformational rearrangement necessary for kifunensine upon binding to the protein explains, in part, why this inhibitor has a higher affinity for the protein than 1-deoxymannojirimycin (32). The additional hydrogen bond interactions between the fused five-membered ring and the protein also contribute to the increased affinity of kifunensine.

Comparison between Human and Yeast α1,2-Mannosidase Structures—Pairwise superimposition of all C-α atoms of the yeast and human α1,2-mannosidase structures yielded a root mean square deviation of 1.44 Å, indicating that the overall structures of both proteins are essentially the same (Fig. 4, A–C). Although differences are observed between the two structures (Fig. 4A), these do not affect the positions of either of the critical active-site residues that are conserved in all class I α1,2-mannosidases or the calcium ion (Fig. 5B). Given the similarities in the structures and the lack of conformational changes seen in human α1,2-mannosidase upon inhibitor binding, it is reasonable to assume that both inhibitors would bind to yeast α1,2-mannosidase in a manner similar to that found for the human protein. In the yeast α1,2-mannosidase crystal structure, an N-glycan from one protein molecule interacts with a symmetry-related protein molecule in what is believed to be an enzyme-product complex (29). Comparison of the yeast α1,2-mannosidase and HM-dMNJ or HM-KIF structures reveals that 1-deoxymannojirimycin should bind to yeast α1,2-mannosidase, whereas steric hindrance would occur between the five-membered ring of the kifunensine molecule and the middle-arm Man\(^\text{a}^\text{2.18} \) of the N-glycan. These observations support the experimental data. Yeast α1,2-mannosidase crystals dissolve immediately when low concentrations of kifunensine are added to the mother liquor, but remain stable when soaked in high concentrations of 1-deoxymannojirimycin. Although a structure of yeast α1,2-mannosidase complexed with 1-deoxymannojirimycin has recently been determined, attempts to co-crystallize yeast α1,2-mannosidase with kifunensine have systematically failed.

In the yeast α1,2-mannosidase structure, a glycerol molecule was found at the bottom of the active-site cavity (29). The structural comparison reveals that the glycerol molecule superimposes with the O-6, C-6, C-5, C-4, and O-4 atoms of the inhibitor molecules (Fig. 5B). This observation reinforces the hypothesis that glycerol mimics saccharide binding (62) and supports the previous conclusion that the glycerol molecule partly occupies the putative binding site for Man\(^\text{a}^\text{2.26} \) (29) (Fig. 5A).

Taken together, these observations strongly suggest that the positions of the six-membered rings of the inhibitor molecules mimic the location of the mannose residue cleaved during the catalytic mechanism. The interaction of the O-2' and O-3' hydroxyl groups of the mannose residue with the calcium ion, the interaction of the O-6' hydroxyl with the strictly conserved residues Arg597 and Glu502, and the non-bonded interactions of C-4, C-5, and the C-6 methylene with Phe659 would almost certainly restrict the orientation of the terminal mannose at the bottom of the active site. The excellent steric complementarity between the inhibitors and the active-site cavity (Fig. 3D) also supports the proposal and suggests that the C-1 atoms of kifunensine and 1-deoxymannojirimycin are in positions homologous to C-1 of Man\(^\text{a}^\text{2.18} \). The C-1 atoms of the HM-KIF and HM-dMNJ structures are ~2.18 and 2.26 Å, respectively, from the O-2' hydroxyl of Man\(^\text{a}^\text{2.18} \) found in the yeast α1,2-mannosidase structure. A flattening of the Man\(^\text{a}^\text{2.26} \) ring structure at C-1 into a skewed-boat conformation and/or distortion of the glycosidic linkage would enable the covalent linkage between C-1 of Man\(^\text{a}^\text{2.18} \) and O-2 of Man\(^\text{a}^\text{2.26} \) to be made without any other significant rearrangements being necessary (Fig. 5C).

In other inverting glycosylhydrorases such as cellulase EG1,

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2 F. Vallée, A. Herscovics, and P. Lynne Howell, unpublished results.
**FIG. 4.** Sequence and structure comparison of human and yeast ER class I α1,2-mannosidases. A, amino acid sequence and secondary structural alignment of yeast (YM) and human ER class I α1,2-mannosidases. The secondary structure elements are colored and labeled as described in the legend to Fig. 2. The sequence alignment was performed with ClustalW Version 1.7 (73). B and C, superimposition of the structures of yeast (yellow) and human (blue) class I α1,2-mannosidases. The structures are shown looking down the (αa)-barrel axis from the long connection (B) and at 90° to the first orientation (C). The following human residues are referred to under “Results and Discussion” (the equivalent yeast numbering is in parentheses): Glu330 (Glu132), Asp463 (Asp275), Arg461 (Arg273), Ser464 (Ser276), Glu467 (Glu279), Leu525 (Leu338), Glu570 (Glu399), Arg597 (Arg433), Glu599 (Glu435), Glu602 (Glu438), Phe659 (Phe499), Glu663 (Glu503), Glu689 (Glu526), and Thr688 (Thr525).
it has been suggested that the protein has evolved to optimally bind the transition state (63). The close complementarity found between inhibitor and protein, the lack of conformational change in the protein upon inhibitor binding, and the similarity of the water molecule positions to the hydroxyls of the inhibitors all seem to suggest that the class I \(a_1,2\)-mannosidases have also evolved to optimally bind the transition state rather than the substrate. The distortion of the substrate to the \(1C_4\) conformation would appear to be dictated by its interactions with the protein and the shape of the active-site cleft, which is preformed prior to saccharide binding.

**Catalytic Residues and Role of Calcium in the Catalytic Mechanism**—The \(a_1,2\)-mannosidase is an inverting glycosylhydrolase that specifically cleaves the \(a_1,2\)-oligosaccharide linkage between C-1 of Man\(^{10}\) and O-2 of Man\(^{7}\) (Fig. 5A) with inversion of the anomic configuration at C-1 and formation of a hydroxyl group at O-2. The catalytic mechanism usually involves two carboxylic acids separated by a distance of \( \approx 9.5 \text{ Å} \), one acting as a general base removing a proton from water and the other acting as a general acid donating a proton to the leaving group. Given the geometrical constraints expected for suitably positioned catalytic residues in inverting enzymes, the yeast \(a_1,2\)-mannosidase structure suggests that the only carboxylic acid groups that could be involved in the catalytic mechanism are Glu\(^{132}\), Asp\(^{275}\), and Glu\(^{35}\) in the yeast enzyme. These correspond to Glu\(^{330}\), Asp\(^{463}\), and Glu\(^{599}\) in the human structure (29). These residues are also the only candidates for catalytic residues in the human protein (Fig. 5B). From the positions of the inhibitor molecules in the HM-KIF and HM-dMNJ structures, it is clear that Asp\(^{393}\), Glu\(^{397}\), Glu\(^{602}\),
Glu$^{663}$, Glu$^{657}$, and Glu$^{689}$ cannot be directly involved in catalysis. These residues either are completely buried at the bottom of the catalytic site or interact with atoms on the inhibitor at some distance from the anomeric C-1 atom (Fig. 3, B and C).

The structure of yeast α1,2-mannosidase enabled two hypotheses regarding the catalytic mechanism to be proposed (29). Please note that the numbering used through out this report is that of human ER class I α1,2-mannosidase (14). Please see the legend of Fig. 4A for the equivalent yeast residues. In the first hypothesis, Glu$^{330}$ was suggested to be the catalytic base, abstracting a proton from a water molecule, which, in turn, attacked the C-1 atom. The identification of the catalytic acid was not possible due to the lack of direct interactions between the O-2' hydroxyl of Man$^7$ and any protein residue. Asp$^{463}$ and Glu$^{599}$ were the most likely candidates, as they are on the opposite side of the glycosidic linkage to be cleaved to Glu$^{330}$ at distances of 9.5 and 9.6 Å, respectively. Asp$^{463}$ was suggested to be the more attractive candidate for the catalytic acid, as it is closer to O-2 of Man$^7$ than Glu$^{599}$. The second, less favored hypothesis implicated Glu$^{599}$ as the catalytic base. Glu$^{599}$ is hydrogen-bonded to a water molecule, which, in turn, is hydrogen-bonded to the glycerol molecule and calcium ion. In this instance, Glu$^{330}$ was suggested to be the most likely candidate for the catalytic acid, as Asp$^{463}$ is on the same side of the oligosaccharide as Glu$^{599}$.

Inspection of the HM-KIF and HM-dMNJ models shows that the six-membered ring structures of both inhibitors are in a C$^4$ conformation. Given this ring pucker, if Glu$^{330}$ is the catalytic base, then attack by a water molecule would result in retention, not inversion, of the anomeric configuration at C-1. Consequently, the attack by water must come from the other side of the inhibitor/substrate molecule, making Glu$^{599}$ and Asp$^{463}$ the only candidates for the general base. If Glu$^{599}$ acts as the base, the only appropriately positioned water molecule on this side of the inhibitor to act as the nucleophile would be Wat$^5$ (Fig. 5B). This water molecule is hydrogen-bonded not only to Glu$^{599}$, but also to the calcium ion (Fig. 3, B and C), and is at a distance of 3.4 Å from the anomeric C-1 atom. This distance could, however, be longer if the Man$^{10}$ saccharide undergoes distortion to a skewed-boat conformation and/or distortion of the glycosidic linkage, as could occur when the Man$^{10}$ saccharide is covalently linked to Man$^7$. Given Glu$^{599}$ as the base, the most likely candidate for the catalytic acid is Glu$^{330}$, as Asp$^{463}$ is on the same side of the inhibitor/substrate as Glu$^{599}$. Glu$^{330}$ is −4.4 Å from O-2 of Man$^7$ and therefore too far away from O-2 for direct attack. A water molecule, Wat$^6$, however, bridges between the carboxylate of Glu$^{330}$ and O-2 of Man$^7$. Given that no significant conformational change is seen upon inhibitor binding, it is hard to envisage that a large conformational change occurs during catalysis. This would suggest that a second water molecule is involved in the catalytic mechanism acting as the acid (Fig. 5D). Although unlikely because it is on the same side of the inhibitor/substrate as Glu$^{599}$, Asp$^{463}$ could alternatively act as the catalytic acid. This residue, like Glu$^{330}$, does not directly hydrogen bond to O-2 of Man$^7$, but interacts via a water molecule.

An alternative mechanism could be envisaged with Asp$^{463}$ as the catalytic base. A water molecule, Wat$^5$, is seen in almost the same position in both inhibitor structures to hydrogen bond to Asp$^{463}$ and the O-2' hydroxyl of the inhibitor molecule (Figs. 3 (B and C) and 5B). In the HM-KIF structure, this water molecule also hydrogen bonds to N-9. This water molecule is 3.44 and 3.78 Å from the anomeric C-1 atom in the HM-dMNJ and HM-KIF structures, respectively. This distance could potentially decrease if the proposed distortion of Man$^{10}$ to the skewed-boat conformation and/or deformation of the glycosidic linkage occurred. If Asp$^{463}$ were the catalytic base, Glu$^{330}$ would be the best candidate for the catalytic acid, as it is on the opposite side of inhibitor/substrate to Asp$^{463}$. As described above, if Glu$^{330}$ were the catalytic acid, an additional water molecule would have to be involved in the mechanism, as Glu$^{330}$ is not within hydrogen-bonding distance of O-2 of Man$^7$, and no large conformational changes are expected to occur during catalysis. In this mechanism, calcium is not directly involved in the catalytic mechanism, but would solely be involved in stabilizing the conformation of the Man$^{10}$ saccharide through its interactions with the O-2' and O-3' hydroxyls. Similarly, in this mechanism, Glu$^{599}$ would only be involved in stabilizing the O-6' hydroxyl of the inhibitor rather than having a dual role and also acting as the base.

Although there remains some ambiguity in the identity of the catalytic acid/base, it is obvious from the inhibitor structures and the yeast α1,2-mannosidase enzyme-product complex (29) that the catalytic mechanism must deviate from the classical inverting enzyme mechanism. As no large conformational changes in the protein are expected during catalysis, and no acidic group is within hydrogen-bonding distance of O-2 of Man$^7$, it would appear that a water molecule must play the role of the acid (Fig. 5D). The first of the two hypotheses also invokes a direct role for the calcium ion in the catalytic mechanism, as the water molecule activated by the putative catalytic base is coordinated by the calcium ion (Fig. 3, B and C). Although not common, calcium has been suggested to activate a water nucleophile in the catalytic mechanisms of inosineuridine N-ribohydrolase (64) and staphylococcal nuclease from Staphylococcus aureus (65). Although there is no direct experimental evidence that the mode of catalysis for this glycosylhydrolase is different from that of other inverting enzymes, deviations in the classical mechanism have been observed for other enzymes (66).

Specificity and Inhibition of Class I α1,2-Mannosidase—Calcium is essential for the activity of class I α1,2-mannosidases (1–5, 17). The HM-KIF and HM-dMNJ structures provide the first direct evidence of the role of calcium in substrate binding. The calcium ion coordinates to the O-2' and O-3' hydroxyls of the inhibitors and appears to help stabilize the C$^4$ conformation of 1-deoxymannojirimycin. In addition, the equatorial conformation of the C-5-C-6 linkage is stabilized by the association of the O-6' hydroxyl with Glu$^{599}$ and Arg$^{397}$ in a side pocket of the active-site cleft (Fig. 3D) and by the van der Waals interactions that the C-6 methane and C-5 and C-4 atoms make with Phe$^{659}$. These interactions appear to be the most critical for inhibitor/substrate binding and help define the specificity of this class of enzymes.

Class I α1,2-mannosidases do not cleave α1,3- or α1,6-linked mannose residues (1–5). From the yeast α1,2-mannosidase enzyme-product complex, it is obvious that an α1,3- or α1,6-linked mannose would not be able to bind to the protein in the same location as kifunensine and 1-deoxymannojirimycin. The difference in the orientation of the saccharide ring when linked to the M7 mannose Man$^7$ through an α1,3- or α1,6-linkage would result in numerous steric clashes between the protein and the oligosaccharide. The inability to cleave α1,3- or α1,6-linked mannose residues will also be determined by the oligosaccharide’s ability to interact with all the protein’s saccharide-binding sites. The enzymes are also specifically inhibited by 1-deoxymannojirimycin and kifunensine, but not by 1-deoxynojirimycin, castanospermine, swainsonine, or 1,4-dideoxy-1,4-imino-D-mannitol. Since the only difference between 1-deoxymannojirimycin and 1-deoxynojirimycin is the orientation of the O-2' hydroxyl (Fig. 6), the specificity of the enzyme for these inhibitors would appear to be dictated by the inhibitor’s ability to coordinate to the calcium ion. Calcium
would be unable to coordinate the O-2' hydroxyl in 1-deoxynojirimycin and therefore cannot stabilize the inhibitor in a 1C4 conformation. The O-2' and O-3' hydroxyls of castanospermine are in the same orientation as those of 1-deoxynojirimycin, suggesting that this inhibitor is also unable to coordinate the calcium ion. These observations confirm the critical role of the O-2' hydroxyl.

The necessity for the substrate or inhibitor to coordinate the calcium ion and the geometry of the active site, especially the side pocket for the C-6–O-6' atoms, also explain the lack of inhibition of class I α,1,2-mannosidases by swainsonine and the poor inhibition by 1,4-dideoxy-1,4-imino-D-mannitol (67) (Table III). Swainsonine and 1,4-dideoxy-1,4-imino-D-mannitol are potent inhibitors of the class II mannosidases such as Golgi α-mannosidase II and lysosomal α-mannosidase (68). In contrast to kifunensine, 1-deoxymannojirimycin, castanospermine, and 1-deoxynojirimycin, which are pyranose mannose and glucose substrate mimics, the class II mannosidase inhibitors swainsonine and 1,4-dideoxy-1,4-imino-D-mannitol are thought to mimic the ring-flattened transition state mannosyl cation of these retaining enzymes as furanose analogs of mannose (68) (Fig. 6). The energy-minimized structures of swainsonine and 1,4-dideoxy-1,4-imino-D-mannitol show that although the furanose ring will have a different pucker to the 1C4 conformation of the pyranose ring of 1-deoxymannojirimycin and kifunensine, the O-2' and O-3' hydroxyls are in the same orientation in all four inhibitors (Fig. 6). This suggests that both swainsonine and 1,4-dideoxy-1,4-imino-D-mannitol should be able to coordinate to the calcium ion. The difference in the structure of 1,4-dideoxy-1,4-imino-D-mannitol and swainsonine explains why the enzyme is inhibited poorly by 1,4-dideoxy-1,4-imino-D-mannitol (67) and insensitive to swainsonine (Table III). Modeling of swainsonine in the active site of human ER class I α,1,2-mannosidase such that the O-2' and O-3' hydroxyls of swainsonine coordinate the calcium ion indicates numerous steric clashes of the pyranose ring with the protein, especially with the strictly conserved Phe659. Unlike the six-membered ring of swainsonine, the C-5/C-6/O-6' arm of 1,4-dideoxy-1,4-imino-D-mannitol is conformationally flexible. The flexibility of this C-5/C-6/O-6' arm would allow the inhibitor to bind to the protein, although the interactions may be less than optimal. This would explain the poor inhibitory properties of this inhib-

### Table III

| Inhibitor                          | IC50 (mM) |
|-----------------------------------|----------|
| Kifunensine                       | 0.2 x 10^-3 |
| 1-Deoxymannojirimycin             | 20 x 10^-3  |
| 1,4-Dideoxy-1,4-imino-D-mannitol  | 1.5      |
| Swainsonine                       | >10      |
| 1-Deoxynojirimycin                | >10      |
| Castanospermine                   | >10      |

*The IC50 values were determined as described under "Experimental Procedures" using a constant 3 ng/µl enzyme and 4 µM Man9GlcNAc2-PA substrate and varying the concentrations of the inhibitor.
The ability of inhibitors to coordinate to calcium appears to be the first and most essential criterion to be considered when designing new inhibitors for class I α,2-mannosidases. The preference for 1-deoxymannojirimycin over 1-deoxyxojirimycin and castanospermine, the lack of inhibition of the enzyme by swainsonine, and the weak inhibition by 1,4-dideoxy-1,4-imino-
D-mannitol all point to the critical role of correctly oriented O-2 and O-3 hydroxyls in a pyranose ring structure, not only for substrate specificity, but also for inhibitor specificity. Other factors that need to be considered include the ability to bind to the protein in the 1C4 conformation rather than the lower free energy 4C1 conformation and the presence of substituents at C-5 capable of forming non-bonded interactions with Ph4659 and hydrogen bonding to Arg299 and Glu999 in the small side
recess of the active site. The pyranose rings of kifunensine and 1-deoxymannojirimycin are identical (Fig. 6). However, kifu-
nensine binds to α,2-mannosidases with higher affinity than 1-deoxymannojirimycin, indicating that the five-membered ring and its substituents are also important components of inhibitor binding and therefore design. The inhibitor structures presented in this study therefore provide the first structural data regarding the specificity of this class of enzymes for α,1,2-
mannosidase residues and a blueprint for future inhibitor design of novel therapeutic agents for the treatment of genetic diseases that are characterized by rapid degradation of misfolded glycoproteins.

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