Structure of *P. citrinum* α1,2-mannosidase reveals the basis for differences in specificity of the ER and Golgi Class I enzymes†.

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**Running title:** Structure of *P. citrinum* α1,2-mannosidase
Abstract

Class I α1,2-mannosidases (glycosylhydrolase family 47) are key enzymes in the maturation of N-glycans. This protein family includes two distinct enzymatically active subgroups. Subgroup 1 includes the yeast and human ER α1,2-mannosidases that primarily trim Man9GlcNAc2 to Man8GlcNAc2 isomer B while subgroup 2 includes mammalian Golgi α1,2-mannosidases IA, IB and IC that trim Man9GlcNAc2 to Man8GlcNAc2 via Man8GlcNAc2 isomers A and C. The structure of the catalytic domain of the subgroup 2 α1,2-mannosidase from Penicillium citrinum has been determined by molecular replacement at 2.2Å resolution. The fungal α1,2-mannosidase is an (αα)7-helix barrel, very similar to the subgroup 1 yeast (1) and human (2) ER enzymes. The location of the conserved acidic residues of the catalytic site and the binding of the inhibitors, kifunensine and 1-deoxymannojirimycin, to the essential calcium ion are conserved in the fungal enzyme. However, there are major structural differences in the oligosaccharide binding site between the two α1,2-mannosidase subgroups. In the subgroup 1 enzymes, an arginine residue plays a critical role in stabilizing the oligosaccharide substrate. In the fungal α1,2-mannosidase this arginine is replaced by glycine. This replacement and other sequence variations result in a more spacious carbohydrate-binding site. Modeling studies of interactions between the yeast, human and fungal enzymes with different Man8GlcNAc2 isomers indicate that there is a greater degree of freedom to bind the oligosaccharide in the active site of the fungal enzyme than in the yeast and human ER α1,2-mannosidases.
Keywords:
Class I α1,2-mannosidases, Golgi apparatus, processing glycosidase, endoplasmic reticulum quality control, binding specificity.

Abbreviations:
dMNJ, 1-deoxymannojirimycin; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum associated degradation; KIF, kifunensine; FM, fungal or *Penicillium citrinum* Class I α1,2-mannosidase; FM•dMNJ, fungal Class I α1,2-mannosidase with bound dMNJ; FM•KIF, fungal Class I α1,2-mannosidase with bound kifunensine; YM, yeast ER Class I α1,2-mannosidase; HM, human ER Class I α1,2-mannosidase.
Introduction

Class I α1,2-mannosidases (Family 47 of the glycosyl hydrolases) participate in different aspects of eukaryotic glycoprotein biosynthesis (for reviews see (3,4)). Although members of this protein family exhibit significant amino acid similarities and characteristic signature motifs (http://afmb.cnrs-mrs.fr/~pedro/CAZY/ghf_47.html), they can be classified into three functionally distinct subgroups according to their enzymatic activities. Subgroup 1 includes the yeast and human endoplasmic reticulum (ER) Class I α1,2-mannosidases that primarily trim Man₉GlcNAc₂ to Man₈GlcNAc₂ isomer B (Fig 1) (5-7). In addition to their role in N-glycan processing, α1,2-mannosidases have also been implicated in ER quality control (for review, see (4)). The second subgroup includes the well-characterized mammalian Golgi α1,2-mannosidases IA (8,9), IB (10-12) and IC (13) as well as α1,2-mannosidases from insect cells (14) and filamentous fungi (15,16). The mammalian enzymes in this subfamily form the Man₅GlcNAc₂ intermediate necessary for the formation of complex and hybrid N-glycans in vivo. This subgroup of enzymes produces Man₈GlcNAc₂ isomers A and C from Man₉GlcNAc₂. The specificity of this subgroup is therefore distinct from that of the ER α1,2-mannosidases (subgroup 1) since the Man₈GlcNAc₂ isomers formed from Man₉GlcNAc₂ are different from the isomer formed by the ER α1,2-mannosidases.

The third subgroup has significant sequence similarity to other members, but does not hydrolyze Man₉GlcNAc₂ (17). Members of this subgroup lack the cysteine residues that form the disulfide bond shown to be essential for yeast α1,2-mannosidase activity (18). This subfamily participates in endoplasmic reticulum associated degradation (ERAD) by
mechanisms that are not yet understood (17,19,20). Overexpression of the mouse and yeast members of this subgroup have been shown to increase the degradation of misfolded glycoproteins by proteasomes. Given their similarity to α1,2-mannosidases, these proteins are proposed to be lectins that recognize high mannose oligosaccharides on misfolded glycoproteins, but additional work is required to elucidate their function.

The ER α1,2-mannosidase from *Saccharomyces cerevisiae* has served as a prototype to define structure-function relationships of Class I α1,2-mannosidases (for reviews see (3,4)). The yeast α1,2-mannosidase structure was determined by X-ray crystallography and shown to be an (αα)₇-helix barrel with one side of the barrel plugged by a β-hairpin (1). The nine highly conserved acidic residues and the calcium ion, all of which are essential for catalytic activity (21), are located at the bottom of a 15Å deep cavity within the barrel. The yeast α1,2-mannosidase is a glycoprotein. In the crystal structure a high mannose oligosaccharide from one molecule extends into the barrel of an adjacent symmetry related molecule interacting with the active site in what is believed to be an enzyme-product complex. This structure has provided information about protein-carbohydrate interactions within the barrel and some insight into the specificity of the ER enzymes. An arginine residue (Arg²⁷³ in yeast) was found to interact with three mannose and one N-acetylglucosamine residues at the branch points of the oligosaccharide. Mutagenesis of this arginine residue to leucine present in mammalian Golgi α1,2-mannosidases produced a mutant enzyme that trims Man₉GlcNAc₂ to Man₅GlcNAc₂. The order of mannose removal by the R273L mutant is different from that of the known naturally occurring subgroup 2 enzymes (22). The structures of the human ER α1,2-
mannosidase ortholog (2) complexed with the inhibitors 1-deoxymannojirimycin and kifunensine and the structure of the yeast enzyme complexed with 1-deoxymannojirimycin (Vallée, Lipari, Sleno, Herscovics and Howell, unpublished results, PDB code 1G6I), reveal no major conformation changes upon inhibitor binding. The inhibitors, with their 2' and 3' hydroxyl oxygen atoms coordinated by the essential calcium ion, are located at the proposed position of the mannose residue cleaved during catalysis. The calcium ion and additional protein-carbohydrate interactions stabilize the mannose analogs in the non-standard \(^1C_4\) conformation. The catalytic mechanism of the \(\alpha1,2\)-mannosidases appears to deviate from the classical inverting enzyme mechanism. Two alternative mechanisms have been proposed, both of which suggest that a water molecule plays the role of the catalytic acid (2).

Although the structural studies of yeast and human ER \(\alpha1,2\)-mannosidases have provided considerable insight into oligosaccharide binding and catalysis, when this work was performed no information was available regarding the structural determinants that account for the different specificities of the two enzymatically active sub-families. The ER (subgroup 1) and Golgi (subgroup 2) \(\alpha1,2\)-mannosidases have complementary actions since the mannose preferentially removed from \(\text{Man}_9\text{GlcNAc}_2\) by the ER enzyme is the last mannose cleaved by the Golgi enzymes (7,12). We present here the structure of a subgroup 2 \(\alpha1,2\)-mannosidase from \textit{Penicillium citrinum} and its complexes with the inhibitors 1-deoxymannojirimycin and kifunensine. The specificity of this enzyme is similar to that of mammalian Golgi and \textit{Trichoderma reesei} \(\alpha1,2\)-mannosidases since it produces \(\text{Man}_8\text{GlcNAc}_2\) isomers A and C from \(\text{Man}_9\text{GlcNAc}_2\) and the last mannose
removed is the mannose preferentially cleaved by the ER α1,2-mannosidases. The structure of the fungal enzyme is an (αα),-helix barrel like the yeast, human and recently determined *Trichoderma reesei* (23) α1,2-mannosidases. There are major structural differences in the carbohydrate binding site of the subgroup I and II enzymes that create a more spacious cavity in the subgroup II α1,2-mannosidases. Comparative modeling of the yeast, human and fungal enzyme interactions with different Man₈GlcNAc₂ isomers indicate that the barrel of the fungal enzyme allows a greater degree of freedom to bind the different forms of the oligosaccharide isomers. In contrast only one conformer of a single isomer, Man₈GlcNAc₂ isomer B, can be accommodated in the yeast and human ER enzymes.
Materials and methods

Expression and purification

Constructs for the expression and methods for the purification of the soluble recombinant fungal α1,2-mannosidase from *Penicillium citrinum* were as described previously in (24). Briefly, the DNA of the *Penicillium citrinum* α1,2-mannosidase gene (*msdC*) (25) lacking its signal sequence (nucleotides 1-105) was cloned downstream of the *Aspergillus* amylase promoter (PamyB) and of the aspergillopepsin signal sequence. The resulting fungal expression vector pTAPM1 was transfected into *Aspergillus oryzae* strain MS2 (argB-). An auxotrophic transformant was cultured in medium containing dextrin as an inducer. The medium was collected after 3 days of culture at 30°C and the recombinant α1,2-mannosidase starting at amino acid 21 was purified from the medium by chromatography on CM-Toyopearl 650M after ammonium sulfate precipitation.

Crystallization, data collection and processing

Crystals of both native protein and protein-inhibitor complexes were grown at room temperature by the hanging-drop vapor-diffusion method. Equal volumes of the protein (20 mg/ml in 10mM NaOAc, pH 5.0) and of the precipitating solutions (17-22% PEG 6K, 50 mM KH₂PO₄, pH 4.6) were suspended over a 0.5 ml reservoir containing the same precipitating solution. Complexes of the fungal α1,2-mannosidase with either 1-deoxymannojirimycin or kifunensine were prepared by co-crystallization. The concentration of each inhibitor (0.5mM to 10 mM) was varied to produce the best crystals. Prism-shaped crystals grew within 2 weeks to a maximum size of 0.6 x 0.4 x 0.3 mm. X-ray diffraction data were collected from the native protein crystals (FM,
space group P2₁, unit cell dimensions a=56.5Å, b=111.0Å, c=86.2Å and β = 99.2°) as well as from the isomorphous crystals grown in the presence of 4 mM kifunensine (FM•KIF) and 10 mM 1-deoxymannojirimicin (FM•dMNJ). Crystals diffracted at room temperature to ~2 Å resolution (Table 1) using CuKα radiation from a Rigaku RU-200 rotating anode generator with a MAR Research (345 mm) image plate detector. All data were processed using DENZO/SCALEPACK (26). Final data reduction statistics are presented in Table 1.

**Structure determination and refinement**

The structure of the native fungal mannosidase was solved by molecular replacement using the CNS software (27). Coordinates of the yeast and human ER α1,2-mannosidases (PDB accession codes 1DL2 and 1FM1, respectively) and a sequence alignment of these proteins with FM were used to create a merged composite computational model of the fungal protein. Loops with poor sequence homology were omitted. In addition, the side chains were modified using the rotamer library in TURBO-FRODO (28) to correspond to the sequence of the fungal protein. This model was used as the search model for molecular replacement. Rotation functions were calculated in the resolution range of 15 - 4 Å. The final molecular replacement solution for the two molecules in the asymmetric unit had a correlation coefficient of 47.5 % and a packing value of 53.7 %. The structure was refined using the maximum likelihood target (29) implemented into CNS (27) with a flat bulk solvent correction and no sigma cutoff applied to the data. Ten percent of the structure factors were randomly selected, excluded from the refinement and used to compute Rfree (30). Refinement of the model using the simulated annealing slow-cooling
protocol (31,32) was alternated with manual inspection and rebuilding of the model using TURBO-FRODO. The two monomers in the asymmetric unit were built independently of each other. The final model for each monomer contains 475 amino acid residues, 1 calcium ion and 9 carbohydrate residues in three N-linked oligosaccharide chains. A total of 201 water molecules obeying proper hydrogen bond geometry were included in the model. The structure comprises amino acid residues 36 to 510. This model was subsequently used to refine the structures of the kifunensine (FM•KIF) and 1-deoxymannojirimycin (FM•dMNJ) inhibitor complexes (Table 1). The initial $\sigma_a$-weighted difference ($F_o - F_c$) electron density maps calculated using the native fungal $\alpha1,2$-mannosidase structure unambiguously showed the location of the inhibitors and calcium ion. Models for 1-deoxymannojirimicin and kifunensine were generated and energy-minimized as described in (2). The FM•KIF and FM•dMNJ structures were refined using the same protocol described above for the native protein. The final refinement statistics for all three structures are presented in Table 1. Analysis of the structures by PROCHECK (33) showed no non-glycine residues in the disallowed regions of the Ramachandran plot.

**Calculations of Connolly surfaces**

Hydrogen atoms were added to the coordinates of the yeast, human and fungal $\alpha1,2$-mannosidases using the SYBYL molecular modeling software package (34) and partial atomic charges were derived using the Pullman procedure. The positions of the hydrogen atoms were optimized using the Tripos force field (35). The Connolly surfaces of the proteins were calculated using the MOLCAD program (36) in the SYBYL software.
Conformational search of the oligosaccharides in the mannosidase binding site

The Man₉GlcNAc₂ oligosaccharide was built in the SYBYL software using the coordinates of the monosaccharides taken from a database of 3D structures of monosaccharides (http://www.cermav.cnrs.fr/databank/monosaccharides/index.html). Atom types and partial charges were defined according to the PIM energy parameters for carbohydrates (37,38) to be used within the Tripos force-field (35).

Nine different complexes were created. For each of the α1,2-mannosidase structures (i.e. yeast, human and fungal), three different orientations of the Man₉GlcNAc₂ substrate were considered. These correspond to the insertion of each branch of the oligosaccharide into the active site of the protein. In each case, the terminal α1,2-linked mannose of the branch was removed, i.e. mannose residues M11, M10 and M9 corresponding to isomers 8A, 8B and 8C, respectively (Fig. 1). The penultimate mannose, M8, M7 or M6, was then oriented in the binding site in the same position as the M7 mannose observed in the binding site of the yeast α1,2-mannosidase structure (1). This corresponds to the +1 site of the catalytic domain. The ensemble consisting of the protein together with this terminal mannose in the +1 site was then considered as rigid and a conformational search of the glycosidic linkages of the oligosaccharide was performed. Three regions were defined as follows: the βMan1-4βGlcNAc1-4βGlcNAc core (yellow in Fig 1) was fixed in an extended conformation, the core oligomannose (mannose residues M3, M4, M5, M6 and M7) was considered as flexible and allowed to undergo a full conformational search,
and the α1,2-linked mannose residues were considered as semi-rigid. In all cases the conformational searches explored the low energy region of each glycosidic linkage as determined in previous conformational studies (37,39,40). The ranges and steps are defined as follows (Fig 1):

for the rigid βMan1-4βGlcNac1-4βGlcNAc core

\[ \Phi_{GN14GN} \text{ and } \Phi_{M14GN} = \Theta(O-5'_C-1'_O-1'_C-4) \text{ fixed at } 280° \]

\[ \Psi_{GN14GN} \text{ and } \Psi_{M14GN} = \Theta(C-1'_O-1'_C-4_C-5) \text{ fixed at } 240°; \]

for the flexible oligosaccharides M3, M4, M5, M6 and M7

\[ \Phi_{M13M} = \Theta(O-5'_C-1'_O-1'_C-3) \text{ from } 50° \text{ to } 90° \text{ in steps of } 10° \]

\[ \Psi_{M13M} = \Theta(C-1'_O-1'_C-3_C-4) \text{ from } 90° \text{ to } 180° \text{ in steps of } 10° \]

\[ \Phi_{M16M} = \Theta(O-5'_C-1'_O-1'_C-6) \text{ from } 50° \text{ to } 90° \text{ in steps of } 10° \]

\[ \Psi_{M16M} = \Theta(C-1'_O-1'_C-6_C-5) \text{ from } 80° \text{ to } 280° \text{ in steps of } 10° \]

\[ \omega_{M16M} = \Theta(O-1'_C-6_C-5_O-5) \text{ two possible values } 60° \text{ and } 300°; \]

and for the terminal part of the branches (semi-flexible)

\[ \Phi_{M12M} = \Theta(O-5'_C-1'_O-1'_C-2) \text{ fixed at } 75° \]

\[ \Psi_{M12M} = \Theta(C-1'_O-1'_C-2_C-3) \text{ from } 90° \text{ to } 180° \text{ in steps of } 30°. \]

The cut-off for allowing inter-penetration of van der Waals spheres was varied between 70% to 87% depending upon how sterically crowded the model was. The hydrogen associated with each hydroxyl was not taken into account during this conformational search and electrostatic interactions were not considered in the energy calculation. The resulting allowed conformations were listed and a clustering program used to define a series of conformational families.
Energy minimization of the complexes

The lowest energy conformation of each family was further optimized in the binding site of the protein. All hydrogen atoms were considered and the electrostatic contribution was included in energy calculations. The whole oligosaccharides as well as the side chains of the amino acids within a sphere of 12 Å radius around the site were fully optimized. The rest of the protein and the calcium ion were included in the calculations as a rigid body.
Results and Discussion

Overall structure of the fungal α1,2-mannosidase

The molecule consists of an (αα)7-barrel composed of 14 helices (α1-α14) which alternate from outside to inside the barrel (Fig. 2). This arrangement of helices results in a topology of seven parallel inner helices and seven parallel outer helices, concentric to the inner helices and anti-parallel to them. The two ends of the (αα)7-barrel are structurally distinct (Fig. 2a,b). On one side, the short-connection or SC side, pairs of inner and outer helices are connected by short loops. This side of the barrel is plugged by the C-terminal β-hairpin. On the opposite side of the barrel, the long-connection or LC side, longer segments that typically contain loops and β-strands connect the helices (Fig. 2b,c). The secondary structural elements of these connections (LC1-7, Fig 2c) typically interact with the loops or strands in the preceding segment (i.e. LC3 with LC2) to cross-link the LC connections. The LC connections are arranged in a circular fashion around the helix barrel and approximately perpendicular to it. The center of the barrel contains a cavity of ~15Å in depth with an average diameter of ~25Å at the level of the β-sheets, decreasing to ~10Å at the top of the C-terminal β-hairpin (all measurements are between C\textalpha positions). The nine highly conserved acidic residues and the calcium ion, all of which are essential for enzyme activity (21), are located at the top of the C-terminal β-hairpin in the center of the barrel. Pairwise superimpositions of the C\textalpha atoms of the fungal structure with those of the yeast and human enzymes yield average root mean square deviations (rmsd) of 1.13 Å for 382 and 1.03 Å for 390 C\textalpha positions, respectively, indicating that the overall structures of α1,2-mannosidase subgroup I and II enzymes are very similar (Fig. 3b,c).
Differences between the fungal and the ER $\alpha_{1,2}$-mannosidase structures include two cis-proline residues, Pro$^{61}$ and Pro$^{234}$, in the loop segments of LC1 and LC4, respectively (Fig 2) and the presence in the fungal enzyme of an extra two-turn helix ($\alpha$X) in LC6 (Figs. 2c and 3a). Two monomers of the fungal protein are present in the crystallographic asymmetric unit. The dimer is formed through interactions between their LC sides with helix $\alpha$X lying at the interface. Twenty-eight residues in each monomer contribute approximately 770 Å$^2$ or 4.3 % of the monomer surface to the dimer interface. The binding of the inhibitors does not perturb the dimer interface. The fungal enzyme is a glycoprotein and the three N-linked oligosaccharides are all located on the LC side of the protein (Fig.2 and 3a). In contrast in the yeast enzyme the N-linked oligosaccharides are located on the SC side.

For the purposes of the following discussion we have divided the cavity of the barrel into two regions; the catalytic/calcium binding site and the oligosaccharide-binding site. The catalytic site is the location at which catalysis occurs, while the oligosaccharide binding site is the protein regions that interacts with the saccharide residues of the different Man$_8$GlcNAc$_2$ isomers.

**Catalytic and calcium binding site**

The positions of the acidic residues required for enzyme activity and of the essential calcium ion are conserved in the fungal enzyme structure. Comparison of the catalytic site residues, Glu$^{122}$, Arg$^{126}$, Asp$^{267}$, Ser$^{268}$, Glu$^{271}$, Arg$^{407}$, Glu$^{409}$, Glu$^{412}$, Glu$^{472}$, Thr$^{501}$, Glu$^{502}$, which are conserved in all Class I $\alpha_{1,2}$-mannosidases, yield for the 11
Cα positions root mean square deviations of 0.56 Å and 0.62 Å between the fungal and yeast and fungal and human structures, respectively (Fig.4). The calcium ion has 8-fold pentagonal bi-pyramidal coordination similar to that observed in the human α1,2-mannosidase structure (2), with all ligands conserved. The calcium is coordinated by the carbonyl oxygen and O-γ atoms of Thr501 and by four water molecules, which are, in turn, hydrogen bonded to one of the carboxylate groups of Glu271, Glu409, Glu412, Glu472. Two additional water molecules complete the coordination. These water molecules are located on the more accessible side of the calcium and are not hydrogen-bonded to any amino acid residue.

Both kifunensine and 1-deoxymannojirimycin bind to the fungal α1,2-mannosidase at the top of the C-terminal β-hairpin located at the bottom of the active-site cavity in the same conformation and orientation seen previously for the structures of the human α1,2-mannosidase complexed with kifunensine (HM•KIF, PDB code 1FO3) and 1-deoxymannojirimycin (HM•dMNJ, PDB code 1FO2) (Fig. 4b). Similar results are also observed for the yeast enzyme complexed with 1-deoxymannojirimycin (unpublished results, PDB code 1G6I). As observed in the human α1,2-mannosidase structures, no significant conformational changes occur in the fungal protein on inhibitor binding (2). However, the two water molecules that completed the calcium coordination are displaced with the calcium interacting instead with the O2'- and O3'- hydroxyl groups of the pyranose ring of the inhibitors. As seen in the human α1,2-mannosidase structures, inhibitor binding appears to stabilize the calcium ion. There is a small decrease in B-factors observed for the calcium ion in both inhibitor complexed structures relative to the
native fungal structure (Table 1). No decrease is observed in the B-factor for the calcium in monomer B of the FM•dMNJ structure (Table 1). The hydroxyl groups of the pyranose rings of both 1-deoxymanojirimycin and kifunensine interact with Arg\textsuperscript{407}, Glu\textsuperscript{409}, Glu\textsuperscript{472}, and Glu\textsuperscript{502} and via water molecules to Glu\textsuperscript{122}, Glu\textsuperscript{412}, Glu\textsuperscript{271}, and Asp\textsuperscript{267}. The O6' hydroxyl group is completely buried in a small side recess of the active site and interacts with Arg\textsuperscript{407} and Glu\textsuperscript{409}. Kifunensine also makes several additional direct and water mediated interactions. The O-7 carbonyl oxygen and N-9 interact directly with Arg\textsuperscript{407} and Asp\textsuperscript{267}, respectively. Water mediated interactions occur between the N-9 and O-γ of Ser\textsuperscript{268}, the O-8 carbonyl and the amide nitrogen of Gly\textsuperscript{273} and the O-9 and N-η2 of Arg\textsuperscript{407}. Calcium ion coordination and an extensive network of hydrogen bonds help stabilize both inhibitors in the non-standard \textsuperscript{1}C\textsubscript{4} conformation. Since the active site conformation and the location of the calcium ion are conserved in the fungal, human and yeast α1,2-mannosidase structures (Fig. 4) it is likely that all Class I α1,2-mannosidases catalyze the cleavage of the α1,2-mannose linkages in the same manner as described in (2).

**Structural differences in the oligosaccharide binding site**

Significant structural differences between the fungal and ER α1,2-mannosidases occur in the oligosaccharide-binding site (see definition of oligosaccharide binding site above and Fig. 5). When the identity of the residues in the carbohydrate binding sites of the fungal and ER enzymes are compared, fewer amino acids (highlighted in red) are conserved between the fungal and the ER enzymes (Fig.5a,b) than between the two ER α1,2-mannosidases (Fig.5c). One of the major differences between the two subgroups of α1,2-
mannosidases is the replacement of an arginine residue, Arg$^{273}$ or Arg$^{461}$ in the yeast and human α1,2-mannosidase structures, respectively, with a glycine in the fungal α1,2-mannosidase (Gly$^{265}$). This arginine was found in the yeast α1,2-mannosidase structure to interact with three mannose residues and one N-acetylglucosamine residue at the branch points of the oligosaccharide (1). The importance of Arg$^{273}$ in conferring substrate specificity was demonstrated by its mutation in the yeast ER α1,2-mannosidase to leucine (22). In addition to this change, the LC3 loop is four residues shorter in the fungal structure (residues 186-195) and as a consequence adopts a different conformation from that observed for the corresponding loop in the yeast and human enzymes. This structural difference together with the replacement in the fungal mannosidase of Arg$^{273}$ with Gly$^{265}$ and the substitution of yeast (human in brackets) residues Glu$^{207}$ (Glu) with Val$^{198}$, Ser$^{204}$ (Thr) with Gly$^{195}$, Asn$^{196}$ (Pro) with Ala$^{191}$, Asn$^{129}$ (Asn) with Ser$^{119}$, Ile$^{254}$ (Met) with Ser$^{246}$ and Tyr$^{255}$ (Phe) with Ser$^{247}$, Arg$^{269}$ (Thr) with Ser$^{261}$ results in the creation of extra space in the carbohydrate binding site and significantly different topology of the protein surface (Fig. 5).

**Modeling studies of Man$_8$GlcNAc$_2$**

To evaluate the importance of the larger cavity in fungal α1,2-mannosidase as a major structural determinant for the different specificities of the subgroup 1 and 2 enzymes, we have modeled the different isomers of Man$_8$GlcNAc$_2$ into the oligosaccharide-binding sites of the fungal, yeast and human α1,2-mannosidase structures. Due to the number of glycosidic linkages that were systematically varied during the conformational search, more than 7x10$^9$ conformations were explored for each of the nine complexes examined.
(see Materials and Methods). The results showed that no allowed conformation could be found for branch A docked into either the yeast or human α1,2-mannosidase nor branch C docked into the yeast α1,2-mannosidase. For all the other complexes, the number of possible oligosaccharide conformations ranged from a few hundred to more than 500,000 depending upon the complex studied and the van der Waals cutoff applied.

Table 2 summarizes the different conformations that the oligosaccharide can adopt in each complex after the clustering and optimization processes. Only the conformations of the central mannose residues (i.e. M4, M5, M6, M7, M8) are given since these glycosidic linkages are the ones that vary the most. Yeast α1,2-mannosidase can only accommodate branch B in its binding site, whereas the human enzyme seems to be able to accommodate both branches B and C. In contrast, each of the branches of the oligomannose can be docked in the fungal α1,2-mannosidase binding site. The number of conformational families predicted to occur is also larger for the fungal enzyme than for either the yeast or human α1,2-mannosidase. All the allowed conformational families listed in Table 2 are displayed in Figure 6.

To validate the docking approach used, the predicted model of branch B of the oligomannose in the yeast α1,2-mannosidase binding site was compared to the conformation observed in the crystal structure (1). In the modeling studies the initial structure of the oligosaccharide was built using a database of 3-dimensional monosaccharide structures (see material and methods) with the terminal mannose fixed in the same position as that seen in the crystal structure. Our calculations predict that only...
one conformation of branch B can fit in the binding site of yeast α1,2-mannosidase, consistent with the B-isomer specificity of this enzyme. There is excellent agreement between the theoretical and experimental values of the torsion angles (Table 2) suggesting that the modeling studies are capable of reproducing the experimental data and that the approach used provides meaningful results.

The results for subgroup 1 enzymes, i.e. yeast and human α1,2-mannosidase, are very similar (Table 2). Only one conformation for the binding of the branch B is found for the human enzyme (Table 2 and Fig. 6 (a)) and with the exception of the ω angle of the M16M-C glycosidic linkage, the torsion angles obtained for both enzymes are essentially the same and in good agreement with the crystal structure. The 136° variation in the M16M-C glycosidic linkage does not appear to change significantly the overall orientation of the C-branch in the binding site (Fig. 6 (b) and Fig.7). In contrast, four different conformational families for branch B bound to the fungal enzyme were found with variations occurring in the orientation of both the M13M-B and the M16M-C linkages (Table 2). In the yeast, human, and fungal enzymes there appears to be only one conformation that the M16M-B linkage can adopt. This conformation is characterized by a small value (< 60°) for the Φ angle.

Much more flexibility is predicted when branches A and C of the oligosaccharide are docked in the binding site of the fungal α1,2-mannosidase (Fig. 6 d,e). For example when branch A is docked at the catalytic +1 site, more than 10 conformational families are predicted to occur, while four families are predicted to occur when branch C is
docked. While two conformational families were also predicted when branch C was
docked in the binding site of the human \(\alpha_{1,2}\)-mannosidase, these are very unlikely
complexes, with high energies, and were therefore not considered further. It is interesting
to note that when the fungal enzyme cleaves Man\(_9\)GlcNAc\(_2\), approximately equivalent
quantities of both Man\(_9\)GlcNAc\(_2\) isomers A and C but no Man\(_8\)GlcNAc\(_2\) isomer B, are
produced (16).

In the yeast \(\alpha_{1,2}\)-mannosidase structure the \(\alpha_{1,2}\)-mannose residues on the A and C
branches were not visible (1). The modeled oligosaccharides allowed identification of
residues involved in binding these \(\alpha_{1,2}\)-mannose residues. The A-branch residues M\(_8\)
and M\(_{11}\) are coordinated by His\(^{71}\) and Met\(^{73}\) in the yeast molecule (Fig.7a, purple
branch). The corresponding residues in the fungal structure, Ala\(^{73}\) and Ser\(^{75}\) (Fig.3a), are
smaller, thus creating more space for oligosaccharide binding. The C-branch residues M\(_6\)
and M\(_9\) (Fig.7a, orange branch) contact residues (corresponding fungal residues are in
brackets) Arg\(^{269}\) (Ser), Glu\(^{399}\) (Asp), Lys\(^{334}\) (Ser), Lys\(^{424}\) (Tyr), Leu\(^{426}\) (Ser), Asp\(^{427}\) (Ser),
Ile\(^{400}\) (Ser). Again, the corresponding fungal residues are smaller than in the yeast
molecule, providing more space for oligosaccharides. The enlarged space explains why a
greater number of conformations of isomer 8B can bind to the fungal protein (Fig.6c) and
also how this contributes to the ability of fungal enzyme to produce isomers A and C
(Fig.7d,e,f).

The models displayed in Fig. 7 are the ones that (i) present the most interesting
contacts between the protein and the oligosaccharide and (ii) allow the reducing GlcNAc
residue to be in an orientation that would be compatible with its attachment to a glycoprotein. The Connolly surface of the protein was calculated and color-coded according to the electrostatic potential. Examination of these surfaces allows for some rationalization of the specificity.

The presence of Arg$^{273}$ at the bottom of the site is a key feature that allows only one precise conformation of branch B in the binding site of yeast and human enzyme. In addition to the change from Arg to Gly, other substitutions mentioned above also help enlarge the active site cavity in the fungal α1,2-mannosidase. The enlarged active site allows for a greater degree of flexibility in the binding of the A and C branches of the oligosaccharide and suggests that there will be less of an entropic penalty when these branches of the oligosaccharide bind to the enzyme then when branch B binds. While the specificity of subgroup I enzymes seems to depend on the presence of very precise interactions between the protein and oligosaccharide substrate, the specificity of subgroup II enzymes appears to be much more subtle relying more on entropic factors and the intrinsic conformational flexibility of the saccharide.

**Access numbers**

The coordinates and structure factors of the *P. citrinum* α1,2-mannosidase and its complexes with 1-deoxymannojirimycin and kifunensine have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org), accession codes XXX, YYY and ZZZ, respectively.
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15, 37-42, 54.


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305-308.

**Figure Legends**

Fig. 1: Schematic representation of the Man$_9$GlcNAc$_2$. The mannose residues are labeled M3 - M11. Removal of mannose residues M11, M10 and M9 results in the formation of Man$_8$GlcNAc$_2$ isomers A, B and C, respectively. Residues on each of the A, B, and C branches are colored purple, red and orange, respectively. The torsion angles varied in the energy calculations and the corresponding glycosidic linkages are labeled according to the type of saccharide unit (M, mannose; GN, N-acetylglucosamine), the chemical linkage ($12, \alpha_1,2$; $13, \alpha_1,3$; $14, \alpha_1,4$; $16, \alpha_1,6$) and the branch they belong to (A, B, C). To distinguish the $\alpha_1,2$ linkages between M11 and M8 from that between M8 and M5 on branch A, the linkage between M11 and M8 is designated M12M-AA.

Fig. 2: Fungal $\alpha_1,2$-mannosidase structure. (a) Schematic ribbon representation of fungal $\alpha_1,2$-mannosidase viewed down the $(\alpha\alpha)$-barrel axis from the LC side. (b) View at 90° orientation to (a). In both (a) and (b), the calcium ion is represented as a blue sphere, the three N-glycans (HM1, HM2 and HM3), the asparagine residues attached to these three N-glycans and one disulphide bridge (S1: Cys332 – Cys361) are shown in ball-and-stick representation. The positions of the cis-prolines are indicated with by initials CP1 and CP2. (c) Topological two-dimensional representation of the fungal $\alpha_1,2$-mannosidase structure. The figure is roughly oriented as in (a). $\alpha$-helices, $3_{10}$-helices and $\beta$-strands are represented as coloured circles, small black circles and arrows, respectively. In all panels the $\beta$-hairpin that plugs the barrel is coloured in pink. The connections between helices on the LC side are labeled LC1-LC7. The secondary structure (see below) was assigned
with the use of the program DSSP (41) and is as follows: \( \alpha_1 \), residues 37-58; \( \beta_1 \), 64-66; \( \beta_2 \), 71-73; \( \alpha_2 \), 81-93; \( \alpha_3 \), 96-107; \( \beta_3 \), 118-119; \( \alpha_4a \), 120-125; \( \alpha_4b \), 127-139; \( \alpha_5 \), 149-166; \( 3_{10a} \), 167-170; \( \beta_4 \), 180-181; \( \beta_5 \), 193-195; \( \alpha_6a \), 196-200; \( \alpha_6b \), 203-213; \( \alpha_7 \), 217-230; \( 3_{10b} \), 235-237; \( \beta_6 \), 247-249; \( \alpha_8 \), 268-279; \( \alpha_9 \), 285-301; \( \beta_7 \), 303-304; \( \beta_8 \), 314-315; \( \beta_9 \), 317-318; \( \beta_{10} \), 323-324; \( \beta_{11} \), 326-328; \( 3_{10c} \), 329-332; \( \alpha_{10} \), 333-345; \( \alpha_{11} \), 348-366; \( \beta_{12} \), 376-378; \( 3_{10d} \), 381-383; \( 3_{10e} \), 386-368; \( \alpha_X \), 389-395; \( \beta_{13} \), 398-401; \( \alpha_{12} \), 409-421; \( \alpha_{13} \), 424-441; \( 3_{10f} \), 456-458; \( \alpha_{14a} \), 468-471; \( \alpha_{14b} \), 473-479; \( \beta_{14} \), 497-499; \( \beta_{15} \), 505-507. In all three panels, N and C indicate the N- and C- termini of the molecule. The colour scheme for the \( \alpha \)-helices, \( 3_{10} \)-helices and \( \beta \)-strands is conserved in all three panels. The figure was prepared with MOLSCRIPT (42) and RASTER3D (43).

Fig. 3: Sequence and structural comparison of fungal, yeast and human \( \alpha_1,2 \)-mannosidase. (a) Amino acids sequence and secondary structural alignment of fungal (FM), yeast (YM) and human (HM) \( \alpha_1,2 \)-mannosidase. The secondary structural elements are colored and labeled as described in the legend of Fig. 2. The position of the N-glycosylation sites in fungal \( \alpha_1,2 \)-mannosidase are labeled HM1-HM3. The sequence alignment was done with Clustal W (44). The figure was prepared using ESPript (45). (b) and (c) Superimposition of the structures of fungal (red), yeast (yellow), and human (blue) \( \alpha_1,2 \)-mannosidase. The structures are shown looking down the (\( \alpha \alpha \gamma \))-barrel axis from the long connection side (a) and at 90° to the first orientation (b). For clarity the N-linked oligosaccharides are not shown.
Fig. 4: (a). Structural superposition of the conserved acidic residues and calcium ions in the active site region of the fungal (red), yeast (yellow) and human (blue) enzymes. Only a subset of the 11 catalytic residues is shown for clarity. Superposition was done with LSQMAN (46) using all C\textsubscript{\alpha} atoms. (b). Structural superposition of the FM•dMNJ (pink), FM•KIF (purple), HM•dMNJ (yellow) and HM•KIF (green). Only residues implicated in catalysis are shown. The following fungal residues are shown (the equivalent yeast numbering is in parentheses): Glu\textsubscript{122} (Glu\textsubscript{132}), Asp\textsubscript{267} (Asp\textsubscript{275}), Ser\textsubscript{268} (Ser\textsubscript{276}), Glu\textsubscript{271} (Glu\textsubscript{279}), Glu\textsubscript{409} (Glu\textsubscript{435}), Glu\textsubscript{412} (Glu\textsubscript{438}), Glu\textsubscript{472} (Glut\textsubscript{503}), Thr\textsubscript{501} (Thr\textsubscript{525}), Glu\textsubscript{502} (Glu\textsubscript{526}).

Fig. 5: Connolly surface of the carbohydrate binding site of fungal (a), human (b), and yeast (c) α1,2-mannosidase. Each panel is colour coded according to the degree of residue conservation from red, 100\% conserved, through white 50\%, to blue 0\% conservation. The panels represent the comparison between (a) yeast and fungal, (b) fungal and human, (c) and human and yeast enzymes. In panels (a), (b) and (c) the equivalent residues in the fungal, human and yeast enzymes are labeled, respectively.

Fig. 6: Schematic diagrams showing the allowed conformations of the oligosaccharide in the α1,2-mannosidase binding site. Orthogonal views of the Connolly surface of the (a) yeast, (b) human and (c) fungal α1,2-mannosidases showing the one or more possible conformations of the oligosaccharide with chain B in the binding site. (d) Orthogonal views of the Connolly surface of the fungal α1,2-mannosidase showing the possible conformations of the oligosaccharide with chain A in the binding site. (e) Orthogonal views of the Connolly surface of the fungal α1,2-mannosidase showing the possible
conformations of the oligosaccharide with chain C in the binding site. In the central column of each panel the allowed conformations of the N-glycan and calcium ion are shown in the absence of the protein surface. The N-glycan is drawn in stick representation and is colored as in Fig 1.

Fig. 7: Connolly surface of the binding site of the α1,2-mannosidases colour-coded as a function of the electrostatic potential (from blue for negative to red for positive values). Selected conformers of the docked oligosaccharides are drawn in stick representation, colored as in Fig 1 and denoted in Table 2 for (a) yeast α1,2-mannosidase chain B; (b) human α1,2-mannosidase chain B; (c) fungal α1,2-mannosidase chain B; (d) fungal α1,2-mannosidase chain C; (e) fungal α1,2-mannosidase chain A conformer A; (f) fungal α1,2-mannosidase chain A conformer B. The numbering of the residues in each panel corresponds to the numbering for the enzyme presented (i.e. yeast, human or fungal). A comparison of the relative numbering for the three enzymes is given in Fig 3a.
Table 1: Data collection and refinement statistics

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a The values given in parentheses are the completeness and $R_{sym}$ for the last resolution shell. $R_{sym} = \sum \sum |I_i - \langle I \rangle| / \sum I$, where $\langle I \rangle$ is the average of equivalent reflections and the sum is extended over all measured observations for all unique reflections.

b $R_{cryst} = \sum |F_o| - |F_c| / \sum |F_o|$, where $F_o$ and $F_c$ are the observed and calculated structure factors, respectively. For $R_{free}$, the sum is extended over a subset of reflections (10%) excluded from all stages of refinement.

c Rmsd, root mean square deviation;

d Two monomers are present in the asymmetric unit monomer A (monA) and monomer B (monB).
Table 2: Torsion angles (°) for the different allowed conformations of the oligomannose. Only the values of the central core are given.

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* Denote the conformations displayed in Fig. 7.

5 Grey shading corresponds to the glycosidic linkage that is the most buried in the model.
Structure of P. citrinum α1,2-mannosidase reveals the basis for differences in
specificity of the ER and Golgi class I enzymes
Yuri D. Lobsanov, Francois Vallee, Anne Imberty, Takashi Yoshida, Patrick Yip, Annette
Herscovics and P. Lynne Howell

J. Biol. Chem. published online November 19, 2001

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