Understanding Protein Structure-Function Relationships in Family 47 α-1,2-Mannosidases through Computational Docking of Ligands

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**ABSTRACT**  Family 47 α-1,2-mannosidases are crucial enzymes involved in N-glycan maturation in the endoplasmic reticulum and Golgi apparatus of eukaryotic cells. High-resolution crystal structures of the human and yeast endoplasmic reticulum α-1,2-mannosidases have been recently determined, the former complexed with the inhibitors 1-deoxymannojirimycin and kifunensine, both of which bind in its active site in the unusual \( {}^{1}C_4 \) conformation. However, unambiguous identification of the catalytic proton donor and nucleophile involved in glycoside bond hydrolysis was not possible from this structural information. In this work, α-d-galactose, α-d-glucose, and α-d-mannose were computationally docked in the active site in the energetically stable \( \Psi \)-conformation as well as in the \( {^1}C_4 \) conformation to compare their interaction energetics. From these docked structures, a model for substrate and conformation selectivity based on the dimensions of the active site was proposed. α-D-Galactopyranosyl-(1→2)-α-D-mannopyranosyl-α-D-glucopyranosyl-(1→2)-α-D-mannopyranose, and α-D-mannopyranosyl-(1→2)-α-D-mannopyranose were also docked in the active site with their nonreducing-end residues in the \( ^{1}C_4 \) and \( E_4 \) (representing the transition state) conformations. Based on the docked structure of α-D-mannopyranosyl-\( E_4-(1\rightarrow2)-\alpha-D\)-mannopyranosyl, the catalytic acid and base are Glu132 and Glu435, respectively. Proteins 2002;49:125–134.

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Key words: AutoDock; docking; enzyme mechanism; mannosidase; structure-function; substrate conformation; transition state

**INTRODUCTION**

Class I α-1,2-mannosidases (EC 3.2.1.113), which form glycosyl hydrolase Family 47,1 are crucial enzymes in N-glycan synthesis in eukaryotic organisms. Glycoprotein synthesis through the N-glycan pathway begins with the transfer of Glc₃Man₉GlcNAc₂ to dolichyl phosphate to freshly synthesized polypeptides in the endoplasmic reticulum (ER).2-4 α-Glucosidases and α-1,2-mannosidases of the ER trim Glc₃Man₉GlcNAc₂ to form Man₉GlcNAc₂, which is transported to the Golgi apparatus for further processing. Subsequent trimming by the Golgi Class I α-1,2-mannosidases results in Man₉GlcNAc₂ formation, which is necessary for the maturation of the N-glycan to hybrid and complex oligosaccharides. ER α-1,2-mannosidase is also involved in the quality control of newly synthesized proteins by indirectly supplying the signal necessary for targeting misfolded proteins for degradation.5,6

The crystal structure of the catalytic domain of ER Class I α-1,2-mannosidase of Saccharomyces cerevisiae has been recently determined.7 It has an unusual (α,α)-barrel structure, with an N-glycan (Fig. 1) from one molecule extending into the barrel of the adjacent symmetry-related molecule, interacting with the enzyme active site. A C-terminal β-hairpin protrudes into the center of the barrel from one side, plugging it. The other side of the barrel has a ~25 Å-diameter funnel-shaped cavity that decreases to ~10 Å at the funnel tube, which is also plugged by a Ca²⁺ ion. Site-directed mutagenesis of Arg273, located in the funnel neck in the yeast enzyme, to Leu273 (all residue numbering henceforth is based on the yeast enzyme) allowed the enzyme to cleave all four α-1,2-linked mannose residues, rather than just the single residue of the middle arm of Man₉GlcNAc₂.8

Crystal structures of the catalytic domain of human ER Class I α-1,2-mannosidase, both with and without the potent inhibitors 1-deoxymannojirimycin (DMJ) and kifunensine (KIF) (Fig. 2), have also been recently determined.9 Both inhibitors bind to the enzyme at the base of its active site, with the Ca²⁺ ion coordinating and stabilizing O2 and O3 hydroxyls of the six-membered rings of both inhibitors in their \( ^{1}C_4 \) conformations. The overall structures of the yeast and human enzymes are essentially the same. Although the amino acid sequences of the two enzymes are no more than 35% similar, the positions of the amino acid residues that make up the active site in the two crystal structures are also practically identical. The most recently determined α-1,2-mannosidase structures are those of Penicillium citrinum, also complexed with DMJ and KIF,10 and Trichoderma reesei.11 Both these enzymes trim Man₉GlcNAc₂ to Man₉GlcNAc₂. Their active sites therefore should be flexible enough to bind not only Man₉GlcNAc₂ but also all the intermediates between

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it and Man9GlcNAc2, unlike the ER enzyme, which binds just the former. Their overall structures, active-site conserved residues, and Ca\(^{2+}\) binding are similar to those of the ER α-1,2-mannosidases. Their aglycone-binding sites, however, are more spacious, and molecular modeling studies on *P. citrinum* α-1,2-mannosidase proved that this gives a wider ability to bind oligosaccharides.10

This structural data, when combined with the distances of the catalytic acid and base from the glycosidic bond needed for catalysis,\(^{12}\) suggest that the only candidates as catalytic proton donor and nucleophile are Glu132, Asp275, and Glu435.\(^{7,9}\) Because of the absence of a terminal middle-arm mannosyl residue in the crystal structure, however, the two catalytic residues could not be clearly identified. The \(^{1}C_4\) conformation of the inhibitors in the −1 catalytic site in the human enzyme suggests that the terminal mannosyl residue would also adopt the \(^{1}C_4\) conformation. Given this ring pucker, Glu132 would then have to be the catalytic acid for the enzyme to invert product conformation, and hence Asp275 or Glu455 would be the catalytic base.\(^{7,9}\) Because Glu132 is too far away from the glycosidic oxygen atom for direct attack, the water molecule W195 would have to mediate proton donation, suggesting an unusual catalytic mechanism for this enzyme.\(^{9}\)

We have used automated docking of ligands into the active sites of glycosyl hydrolases and the carbohydrate-binding site of surfactant protein D\(^{-19}\) with AutoDock\(^{20-22}\) to predict different bound ligand conformations. Therefore we thought that it would be possible to use computation to supplement the available experimental knowledge of the active-site function and hydrolysis mechanism of Family 47 α-1,2-mannosidases. Docking using AutoDock consists of first generating different conformations of the ligand to cover all its orientational, translational, and torsional degrees of freedom, and then calculating the interaction energy between the ligand and protein to find the lowest-energy conformation. Computational docking has the advantage of not being limited to a thermodynamically stable state, as are experimental techniques to study ligand binding at atomic resolution, such as X-ray crystallography. Therefore this flexibility can be used to choose ligands to infer binding modes for biological macromolecules and sometimes enzyme reaction mechanisms.\(^{23,24}\) Because there is some ambiguity over the identity of the catalytic base in ER α-1,2-mannosidase, this enzyme is an interesting test case for docking studies.

In the present study, DMJ and KIF in the \(^{1}C_4\) conformation were first docked to validate that AutoDock would yield the same bound structures as found by X-ray crystallography. Next DMJ, 1-deoxynojirimycin (DNJ), α-d-galactopyranose (Gal), α-d-glucopyranose (Glc), and α-d-mannopyranose (Man; Fig. 2) were docked both in the \(^{4}C_1\) conformation, favored by these molecules in solution, as well as in the \(^{1}C_4\) conformation to compare the binding energetics of the two conformations with the active site. To understand the nature of transition-state stabilization in α-1,2-mannosidase, the disaccharides α-d-galactopyranosyl-(1→2)-α-d-mannopyranose (Gal-1,2-Man), α-d-glucopyranosyl-(1→2)-α-d-mannopyranose (Glc-1,2-Man), and α-d-mannopyranosyl-(1→2)-α-d-mannopyranose (Man-1,2-Man) (Fig. 2) were docked into the active site. Each of these ligands was docked two ways, with the nonreducing-end residue either in the \(^{4}C_1\) or the \(E_4\) (representing the transition state) conformations.

**COMPUTATIONAL METHODS**

**Multiple Sequence Alignment**

Multiple sequence alignment followed by mapping onto the tertiary structure of yeast α-1,2-mannosidase was accomplished using the MSA3D tool of Protein Explorer.\(^{25}\) In calculating conservation, the amino acid residues were divided into several groups based on chemical properties and structural similarity of their side chains as follows: alanine/isoleucine/leucine/methionine/valine, phenylala-
nine/tryptophan/tyrosine, arginine/histidine/lysine, aspartic acid/glutamic acid, serine/threonine, and asparagine/glutamine. Glycine, proline, and cysteine were not grouped with any other amino acid because of the unique roles they play in a protein’s folding into its tertiary structure. A residue had to be 100% identical or similar to be considered conserved.

Automated Docking

AutoDock employs a Lamarckian genetic algorithm (LGA) to perform a search of the conformational space of the ligand. The macromolecule is assumed to be rigid, a reasonable assumption in this case since there are very small conformational changes in the structures with and without a ligand in the interior of the active site. In the AutoDock implementation of the genetic algorithm, the genes are a string of real values representing the three cartesian coordinates for the ligand translation, four variables for the quaternion defining the ligand orientation, and one real value for each ligand torsion, in that order. Search of the ligand conformational space begins by generating a population of random conformers that uniformly covers the grid space (a grid-based method is used for energy evaluations, and interaction energies at every point in the grid surrounding the protein are precalculated for efficiency). This is followed by a loop over the number of generations, wherein the population of each generation is subjected to mutation, crossover, and a local search.

The LGA uses a local search algorithm from Solis and Wets (SW) after the global search performed by the genetic algorithm, and the local search results are inherited by the offspring. This search algorithm is adaptive; the step size is modified depending on the recent energy history. User-defined numbers of consecutive increases or decreases in the energies cause the step size to be doubled or halved, respectively. A slightly modified version of the SW method that allows different step sizes for different genes has also been implemented in AutoDock.

The yeast α-1,2-mannosidase crystal structure (PDB 1DL2) was chosen for this docking study. All hydrogen atoms in both protein and ligands were explicitly modeled, with polar hydrogen atoms being assigned Lennard-Jones 12-10 hydrogen bonding parameters and nonpolar hydrogen atoms being assigned 12-6 parameters. Hydrogen atoms were added to structure 1DL2 using CHARMM. All water molecules were removed while docking. Partial charges were assigned to the protein atoms using all-atom charges of the AMBER force field. Atomic solvation parameters and fragmental atom volumes were added using the AddSol program provided in the AutoDock 3.06 suite.

In the testing phase of the study, DMJ and KIF structures were first isolated from the crystal coordinates of human α1,2-mannosidase. Hydrogen atoms were added using Babel and partial charges for inhibitors and mannosyl substrates were generated using MOPAC. Rotatable ligand bonds were defined using the AutoTors module of AutoDock.

The grid maps for van der Waals and electrostatic energies were prepared using AutoGrid version 3.0, with 101 × 101 × 101 points spaced at 0.375 Å distances. The grid was centered on the Ca2+ ion at the base of the active site. AMBER force-field parameters were used for evaluating nonbonded interaction energies. The Ca2+ parameters were the same as those used by Allen et al. Electrostatic interactions were evaluated using a distance-dependent dielectric constant to model solvent effects.

For the LGA, the size of the initial random population was 50 individuals, the maximal number of energy evaluations was 1.5 × 10⁶, the maximal number of generations was 80, the number of top individuals that survived into the next generation, the elitism, was 1, the probability that a gene would undergo a random change was 0.02, the crossover probability was 0.80, and the average of the worst energy was calculated over a window of 10 generations.

The pseudo-SW method was used for local searches. There were a maximum of 300 iterations per local search, the probability of performing a local search on an individual was 1.0, the maximal number of consecutive successes or failures before doubling or halving the step size of the local search was 4, and the lower bound on the step size, 0.01, was the termination criteria for the local search. A total of 100 dockings were performed in each docking run. In analyzing the docked conformations, the clustering tolerance of the root mean square positional deviation was 1.0 Å. The crystal coordinates of DMJ and KIF were references for their docking. For disaccharide dockings, crystal coordinates of DMJ and M7 (Fig. 1) served that purpose.

The involvement of two crystal-structure water molecules, W54 and W195, in the catalytic mechanism for nucleophilic attack and mediation of proton donation, respectively, has been suggested. Because these waters could be displaced upon substrate entry, they were optimized individually with docked Man-E1,2-Man already in place using the local search algorithm of AutoDock. The minimization parameters were the same as those used for the LGA local search.

The main aim of this docking study was to identify the catalytic residues by determining the bound conformation of a mannosyl residue in the tube of the active-site funnel, so a local search of the conformational space inside this tube was needed. However, a global search was expected to yield interesting results also. Therefore, to suit our specific docking needs we increased the local search character of the LGA by making the probability of local search 1.0, and reduced the global search character by keeping the maximal number of generations over which the genetic algorithm is looped to 80. Also, to reduce the search of meaningless conformational space, the initial conformation of the docked ligand was placed in the active-site funnel tube by superimposing it on the DMJ or KIF crystal coordinates.

All docking jobs were run on an SGI Origin 2000 with a 300-MHz MIPS R12000 processor and 1 GB of memory running IRIX 6.5.
RESULTS

Multiple Sequence Alignment

The following eleven typical α-1,2-mannosidases underwent multiple sequence alignment, with their Swiss-Prot accession numbers in parentheses: murine IA and IB (P45700, P39098), human IA and IB (P33908, O60476), Oryctolagus cuniculus (P45701), Sus scrofa (O02773), Drosophila melanogaster (P53625), Spodoptera frugiperda (O18498), P. citrinum (P31723), Aspergillus phoenicis (Q12563), and S. cerevisiae (P32906). The regions of complete conservation in the family are mapped onto the crystal structure of yeast α-1,2-mannosidase.7 Figure 3 shows that solvent-exposed residues in this enzyme family are strictly conserved only in the active-site funnel tube and neck. The enzyme interior also is highly conserved. The 15 fully conserved residues in the active site are Phe131, Glu132, Ile135, Arg136, Asp275, Ser276, Glu279, Arg433, Pro434, Glu435, Glu438, Phe499, Glu503, Thr525, and Glu526.

Automated Docking

Validation of the docking procedure with inhibitors

The first step was to validate our method by docking DMJ and KIF in the active site of the yeast crystal structure to check for agreement with the observed structures. Because the two enzyme active sites are practically the same, human α-1,2-mannosidase was superimposed on the yeast structure to obtain the corresponding positions of KIF and DMJ in the yeast enzyme, and these coordinates were used to compare the docked structures. The lowest-energy docked structure of KIF-1C4 (KIF in the 1C4 conformation) has a final docked energy of −107.0 kcal/mol; it docked with a root mean square deviation (RMSD) of 0.72 Å to the crystal structure (Fig. 4, Table I). Similarly, docking of DMJ-1C4 gave a final docked energy of −95.0 kcal/mol and an RMSD of 0.62 Å from the crystal structure (Fig. 4, Table I). The agreement was sufficiently good to proceed with the docking of other molecules into the enzyme active site.

DNJ, a Glc analogue, does not inhibit α-1,2-mannosidase.9 As a negative control, DNJ-1C4 was docked into the enzyme active site to compare its docking energy with that of DMJ. The lowest-energy docked structure of DNJ-1C4 has a total docking energy of −88.5 kcal/mol with a 1.31-Å RMSD of DMJ-1C4. This energy is higher than the docking energy of DMJ-1C4 and apparently is not sufficient to compensate for the loss of entropy and solvation energy incurred by it upon binding; that is, its free energy of binding is not negative.

Monosaccharide docking simulations

The lowest-energy docked structure of Man-1C4, with an energy of −93.0 kcal/mol, does not dock in the active-site funnel tube. Instead, it docks just outside the funnel neck away from M7. This structure hydrogen-bonds Asp61, Trp82, Arg136, and Glu497. Of these, only Arg136 is conserved, implying that this binding site is not unique across the α-1,2-mannosidase family. However, the second lowest-energy cluster docks very close to the crystal-structure DMJ-1C4, with a RMSD of 0.82 Å and an energy of −84.3 kcal/mol (Table I). The lowest-energy cluster of Man-4C1 docks close to M7 with an energy of −94.6 kcal/mol (Fig. 5). The second lowest-energy cluster docks in the funnel tube with an energy of −88.5 kcal/mol and a
RMSD of 1.87. Glc and Gal, C2 and C4 epimers of Man, respectively, bind with higher energies than DMJ-1\textsuperscript{C4}.

**Disaccharide docking simulations**

The $E_4$ conformation of Man-1,2-Man docks with a lower energy than the $^1C_4$ conformation; this is not the case in the docking simulations of Gal-1,2-Man and Glc-1,2-Man (Table I). Only Man-$E_4$-1,2-Man can closely overlay the crystal structures of both DMJ and M7 in the active site (Fig. 4), as is evident from their low RMSD values (Table I).

**DISCUSSION**

**Multiple Sequence Alignment**

The alignment covers eukaryotic mammalian, insect, and fungal sequences that are widely separated by evolution. Because the crucial function these enzymes play in posttranslational modifications puts them under great evolutionary pressure, only the critical residues in this enzyme family seem to have been conserved by evolution. Both ER and Golgi enzymes are represented in this
alignment; hence, the conserved residues of the tube and neck should be responsible for binding both nonreducing-end (−1Man) and reducing-end (+1Man) mannosyl residues.

The conserved residues of the active-site neck that bind +1Man are shown in Figure 6. Conserved residue Asp275 hydrogen-bonds the O3 and O4 atoms of +1Man, thus securely anchoring the substrate for the reaction to occur. At most one hydrogen bond can form if −1Man is linked by α-1,3, α-1,4, or α-1,6 bonds to +1Man, thus explaining its specificity for α-1,2-linked mannosyl residues. Because the active site widens at the +1 site, steric hindrance for other than α-1,2-linkages is unlikely. This might explain why the D275N mutant of the S. cerevisiae enzyme has very low α-1,2-mannosidase activity compared to the wild-type enzyme.32

Hydrophobic packing interactions between conserved aromatic residue Phe131 and the C4, C5, and C6 atoms of +1Man and between conserved Leu338 and the C2 and C3 atoms of +1Man also seem to have an important role in stabilizing +1Man (Fig. 6). For a negative-binding free energy, the interaction energy between the ligand and enzyme should compensate for entropy loss and solvation energy loss due to binding. The active site, being rich in acidic amino acid residues, can replace the hydrogen bonding network supplied by water. Binding to the enzyme active site also supplies a hydrophobic environment for the nonpolar carbon atoms of the ligand, and this could be key in causing the negative binding free energy.

### Automated Docking of Inhibitors and Monosaccharides

The very negative docking energies of DMJ-1C4 and KIF-1C4 explain their inhibitory action. The total docked energy in AutoDock is the sum of the total nonbonded intermolecular interaction energy between every ligand atom and the macromolecule and the nonbonded intramolecular energy of the ligand. An interesting point to be noted in these docked structures is the very high interaction energies of the C2 and C3 hydroxyl groups of the inhibitors with the enzyme (Fig. 7). In the lowest-energy docked structure of DMJ, they contribute −25.0 kcal/mol, almost 27.5% of the total interaction energy and 26.3% of the total docking energy of DMJ with the α-1,2-mannosidase. Equivalent values with KIF are −22.7 kcal/mol, 22.7%, and 21.2%. Both these hydroxyl groups are coordinated by the Ca2+ ion present at the base of the active-site funnel tube. Thus, the Ca2+ ion plays a very important role in stabilizing the energetically unfavorable 1C4 conformation of these inhibitors. Another point to be noted is that the activation energy for the transformation of mannos from the 4C1 to the 1C4 conformation is 12−14 kcal/mol, so these interactions alone may contribute significantly to causing the observed ring pucker.

DMJ-4C1 has a much higher docking energy than DMJ-1C4, the difference being higher than the difference in energies of the 4C1 and the 1C4 conformations (−4 kcal/mol), thus making the docked 1C4 conformation more stable. DNJ-1C4 and DNJ-4C1 dock with higher energies than the docked energy of DMJ-1C4, explaining why DNJ is not an inhibitor.

Docked Man-4C1 of the lowest-energy cluster is located away from the active-site neck (Fig. 5), whereas its orientation is the same as that of M7, suggesting how there might be a attractive force on +1Man, pulling it away from the funnel neck and inducing a strain on the scissile bond. The force may also aid in deforming −1Man to the E4 conformation. The docking energy of the second lowest-energy cluster is too high for it to bind favorably in the funnel tube.

### Table I. Inhibitor, Monosaccharide, and Disaccharide Docking

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Conformation</th>
<th>No. of Major Clusters</th>
<th>Cluster Rank</th>
<th>Lowest Energy (kcal/mol)</th>
<th>RMSD of Lowest Energy (Å)</th>
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<td>DMJ</td>
<td>1C4</td>
<td>2</td>
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<tr>
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*Lower-energy clusters that dock outside the active-site funnel tube are ranked but not reported.

1Å RMSD cluster tolerance, based on the crystallographic coordinates of DMJ (inhibitors and monosaccharides) or DMJ and M7 (disaccharides).
Enzymes function by stabilizing the transition state; however, for efficient function the active site should not favorably bind the product. The difference in the binding energies of DMJ-1C4 and Man-1C4 clearly demonstrates how enzymes achieve this kind of specificity. DMJ-1C4 binds the active site with very negative interaction energy, whereas Man-1C4 does not. The hydroxyl group of C1 in Man-1C4 is equatorially oriented and clashes with the CD2 atom of Leu338. This causes a change in the hydrogen bonding energies of the hydroxyl groups of the two ligands, of which the most distinct change is seen in the hydroxyl group of C6. Whereas in DMJ the O6 hydrogen bonds to the OE1 and OE2 atoms of Glu435 with distances of 2.9 Å and 3.1 Å, respectively, the equivalent distances for the O6 of docked Man-1C4 are 3.6 Å and 4.4 Å, too great for hydrogen bonding to occur. The enzyme active site is optimized to bind the E4 conformation of -1Man. Man-E4 in solution has a steric energy of ~10 kcal/mol greater than that of the Man-1C4. Before bond breakage the interactions provided by the rest of the substrate stabilize the E4 conformation and, as suggested earlier, might even induce the strain necessary for its deformation. After the bond breaks, the cleaved mannose probably leaves the funnel tube in an effort to relax back into an energetically more favorable conformation.

Given the orientation of M7, the active-site dimensions (Fig. 8) show that the funnel neck is too narrow for Man-1C4 to enter the -1 subsite. The dimensions indicated in Figure 8 have been obtained by subtracting the van der Waals radii of the two atoms involved from the center-to-center distance of the atoms. At the angle at which measurement has been made, the hydroxyl groups that lie above and below the plane of entry have been excluded from the measurement, as they will not obstruct entry. A hydrogen bond at the neck between the protein and the ligand would modify the dimensions of the neck diameter, as hydrogen bond formation allows closer contact between the atoms involved. However, this would not affect the assertion stated above. The fact that Man-1C4 does dock inside the tube is an artifact introduced by the LGA docking process; mutations allow ligands to explore conformational space that may not be physically available to them. In this case, initial conformer placement inside the tube also helps ligands dock there. Most of the ring substituents of Man-1C4 are equatorial, making the molecule more extended and flat than Man-1C4, which has its ring substituents predominantly in the axial position, thus making it more compact and globular. The active-site
funnel neck is constricted and is just wide enough for Man-1C₄ to squeeze through, while Man-4C₁ cannot (Fig. 8). Two salt bridges on either side of the opening, Glu132–Arg136 and Glu399–Arg433, stiffen the opening and reduce its flexibility. Measuring the active-site opening dimensions in exo-glycosidases might therefore aid in screening out conformations that cannot enter the active site.

There is also the possibility that the neck expands to allow entry of the ligand. However, if the neck of the opening were flexible, its flexibility would be reflected in the temperature factors of the residues of the tube and neck of the active-site funnel in the yeast crystal structure. These residues show very low temperature factors (13–20 Å²) and belong to the helices in the interior of the protein (except for Arg433, which belongs to a loop but which also has low temperature factors). An expansion of the helical core to accommodate the ligand would also be energetically too expensive. Local movement of the side chains of the neck residues seems restricted, as is reflected by their low temperature factors.

Man₈GlcNAc₂, the hydrolysis product of Family 47 ER α-1,2-mannosidases, is a necessary signal for degradation of misfolded protein. α-1,2-Mannosidase inhibitors block degradation of misfolded proteins, and it has been suggested that this slow-acting ER α-1,2-mannosidase ($K_M = 0.5 \text{ mM}$, $k_{cat} = 12 \text{ s}^{-1}$ for the *S. cerevisiae* enzyme$^{32}$) may work as a timer for glycoprotein degradation.$^{5,6}$

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**Fig. 9.** Catalytic mechanism. Putative catalytic residues (green), docked Man-E₁,2-Man (light gray, with nonreducing-end C1 atom in dark gray). Crystal-structure (blue) and minimized water (oxygen in red, hydrogen in white) molecules. Distances in Å. Glu132 is the catalytic acid and Glu435 is the catalytic base.

**Fig. 8.** (a) Active-site neck dimensions compared to (b) those of Man-1C₄ and Man-4C₁. Distances in Å are approximately along the plane of the neck opening.
Two different scenarios can be envisaged here. The first is that the α-1,2-mannosidase selectively binds middle-arm α-1,2-linked \( ^1C_4 \)-mannosyl residues of the N-glycan attached to the glycoprotein. The difference in steric energy between Man-\(^1\)C\(_4\) and Man-\(^3\)C\(_4\) in solution is 4.38 kcal/mol,\(^\text{38}\) which implies that the Man-\(^1\)C\(_4\) solution concentration is <1% of the total Man concentration. Because α-1,2-mannosidase binds the \(^1C_4\) conformation, the corrected \( K_d \) for this enzyme would therefore be ~0.005 mM and \( k_{\text{cat}}/K_M \) would be \( >2.4 \times 10^6 \) M\(^{-1}\) s\(^{-1}\), giving this enzyme very high affinity for its substrate. Also, the relaxation time for the conformational change between the two chair forms is of the order of microseconds.\(^\text{36}\) The rate-limiting step, therefore, seems to be the diffusion of the bulky substrate to the α-1,2-mannosidase active site. The lower concentration of the \(^1C_4\) conformation might be indirectly responsible for the role of the α-1,2-mannosidase as a timer in glycoprotein degradation.\(^\text{5,6}\) One possibility is that selective binding to a less common conformation may be nature’s way of implementing a random selection procedure for quality control.

The second scenario is that the transformation of the terminal α-1,2-linked mannosyl residue from the \(^4C_4\) to the \(^1C_4\) conformation is mediated by interactions with the enzyme, but this seems less likely considering the high activation energy for this transformation.

**Transition State**

Ring flattening at the C1 position of DMJ, leading to the \( E_4 \) conformation, was suggested by Vallée et al.\(^\text{9}\) so that it could form an α-1,2 bond with M7. This would also be consistent with the absolute stereochemical requirement of the coplanarity of C1, C2, C5, and O5 atoms of the nonreducing-end residue necessary for forming the oxocarbenium-ion-like transition state.\(^\text{12}\) The disaccharides Gal-1,2-Man, Glc-1,2-Man, and Man-1,2-Man were docked with the nonreducing-end residues in their \(^1C_4\) and \( E_4 \) conformations. In each case the reducing-end mannosyl residue was in the relaxed \(^4C_4\) conformation. The disaccharide with the nonreducing end in the \( E_4 \) conformation would therefore mimic the substrate transition state.

The steric energy of Man-\( E_4 \) in solution is ~10 kcal/mol higher than that of Man-\(^1\)C\(_4\).\(^\text{38}\) Therefore, for transition-state stabilization to occur, the decrease in energy upon binding should overcome the difference in energy between the two conformations. The lower energy of the docked Man-\( E_4 \)-1,2-Man compared with that of Man-\(^1\)C\(_4\)-1,2-Man clearly establishes that docked Man-\( E_4 \)-1,2-Man well approximates the enzyme-transition state complex. Energies of the two other disaccharide pairs are higher for their \( E_4 \) than for their \(^1C_4\) conformations. This implies that even if Gal-1,2-Man and Glc-1,2-Man penetrate the active site, their \(^1C_4\) conformations will not deform, and hence there will be no substrate activation for hydrolysis.

An examination of the interaction energies of the various substrate hydroxyl groups indicates that three, O3 and O6 of −1Man and O4 of +1Man, form strong hydrogen bonds with the enzyme (interaction energies of 12.0, 12.2, and 13.8 kcal/mol, respectively). This implies that the interactions provided by the hydroxyl groups further away from the scissile bond also contribute significantly to transition-state stabilization. These bonds might also help generate the torque necessary for −1Man deformation.

**Catalytic Mechanism**

Identification of the catalytic acid/proton donor and base/nucleophile from the human and yeast mannosidase crystal structures was somewhat ambiguous.\(^\text{7,9}\) Docking of Man-\( E_4 \)-1,2-Man and the optimal positions of W54 and W195 after minimization clearly establish their identities: Glu132 is the catalytic proton donor and Glu435 is the catalytic base (Fig. 9). Upon optimization, W54 is 0.7 Å and W195 is 0.8 Å from their corresponding crystal coordinates. As suggested earlier,\(^\text{7}\) W195 appears to mediate proton donation, because Glu132 is not within hydrogen-bonding distance of the O2 atom of M7. To the best of our knowledge, this is the first case of a water molecule mediating proton donation from a carboxylic acid. A Grotthuss-type proton hop mechanism involving a channel of water molecules for proton transfer from the proton donor,\(^\text{37}\) in this case a histidine residue, and nucleophilic water\(^\text{38}\) have been suggested before. Another possibility could be movement of Glu132 to within hydrogen-bonding distance of the O2 atom. However, this does not seem likely because Glu132 is anchored in place by a salt bridge with Arg136 that restricts its motion. The water molecule activated by the nucleophile is W54, the only crystal-structure water that is located close enough to the C1 of −1Man for nucleophilic attack. Asp275 is not within hydrogen-bonding distance of W54 and hence is ruled out as the catalytic base (Fig. 9). W54 also is coordinated by the Ca\(^{2+}\) ion; hence Ca\(^{2+}\) has a more direct role in catalysis than stabilizing the \(^1C_4\) conformation of −1Man. Involvement of Ca\(^{2+}\) in activating the nucleophilic water has been observed before.\(^\text{39}\) Given the above mechanism, we can see that KIF does not mimic the substrate transition state. However, it does bind in the active site because of its favorably positioned O2 and O3 hydroxyl groups.

**CONCLUSIONS**

The detailed energetic information supplied by AutoDock significantly adds to the structural information obtained by X-ray crystallography and NMR. This information, upon careful interpretation, can contribute to understanding biomolecular recognition mechanisms. It is evident that, in the postgenomic era, when protein structural information will be available at genomic scale, detailed study of interactions at the atomic level would be invaluable for developing methods to determine a protein’s function from its structure.

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