Protein engineering study of β-mannosidase to set up a potential chemically efficient biocatalyst

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Received on January 22, 2014; revised on July 15, 2014; accepted on July 15, 2014

This study is focused on the analysis and mutagenesis of β-mannosidase from Bacteroides thetaiotaomicron with the aim of broadening its substrate specificity to 2-acetamido-2-deoxy-β-D-mannopyranosyl (β-ManNAc) derivatives. Various conformations (C1, H5 and S2) of native and modified ligands were docked to the binding site of the protein to determine the most suitable conformation of sugars for further hydrolysis. Key amino acid residues were mutated in silico focusing on stabilizing the acetamido group of β-ManNAc as well as forming the oxazoline intermediate needed for hydrolysis. The results of large set of 5 ns molecular dynamic simulations showed that the majority of the active site residues are involved in substrate interaction and do not exhibit a higher flexibility except for Asn178. Mutations of Asn178 to alanine and Asp199 to serine could lead to a stabilization of the acetamido group in the binding site. So far, in vitro mutagenesis and the screen of a large variety of biological sources were unable to extend β-mannosidase’s activity to include β-ManNAc derivatives.

Keywords: β-mannosidase / docking / molecular dynamics / mutagenesis / protein engineering

Introduction

The β-mannosidase from Bacteroides thetaiotaomicron (PDB ID: 2JE8) (Tailford et al. 2007) is an important exo-acting retaining glycosidase hydrolase (GH), belonging to the family 32 of GH2 GHs [the carbohydrate-active enzymes database; Lombard et al. 2014]. This enzyme plays an essential role in the complete hydrolysis of β-mannans to mannose and, therefore, β-mannosidase is very effective in various industrial processes, such as oil and gas drilling or coffee extraction, and as a bleach-boosting agent in the pulp and paper industries (Moreira and Filho 2008). β-Man are also used in the synthesis of oligosaccharides or alkyl β-mannosides for medical and other purposes (Itoh and Kamiyama 1995; Ademark et al. 1999; Nashiru et al. 2001). Clan GH2 contains over 700 sequences encoding enzymes with a wide spectrum of different exo-acting β-glycosidase activities including β-galactosidase (EC 3.2.1.23), β-mannosidase (EC 3.2.1.25), β-glucuronidase (EC 3.2.1.31) and exo-β-glucosaminidase (EC 3.2.1.1).

Glycoside hydrolases have now been classified into over 110 GH families on the basis of sequence similarities providing a rich palette of the conformational landscape of enzymatic glycosylation reactions (Davies et al. 2003). Emerging evidence suggests that many retaining glycosidases that are active on α- or β-mannosides harness unusual B2,5 (boat) transition states. A detailed structural and biochemical study for a boat-like transition state in reported β-mannosidase (B2,5) or closely related conformations confirmed a tight binding of the substrates in the retaining hydrolase mechanism of β-mannosidase (Tailford et al. 2008). Experimental support for catalysis along trajectories passing through boat conformations showed the S2 skew-boat conformation for the Michaelis complex, coupled to the S2 skew-boat conformation for the intermediate. This Michaelis complex displays a classical geometry for the nucleophilic substitution to the anomeric carbon. The structure of β-mannosidase strongly supported the electrophilic migration of the anomeric carbon along the S3→B2,5→S2 itinerary (Offen et al. 2009). Independent ab initio molecular dynamics (MD) studies on non-enzymatic mannosyl oxacarbenium ion interconversion trajectories were entirely consistent with the proposals for enzymatic itineraries during mannoside catalysis (Ionescu et al. 2006).

N-Acetyl-β-D-mannopyranosamine (β-ManNAc) is a precursor of biosynthesis of the neuraminic acids found in glycolipids and glycoproteins. β-N-Acetylmannosaminidase has not been identified in nature so far. So the main goal of our study was to produce β-N-acetylmannosaminidase via the mutagenesis of β-mannosidase. The concept of extending the β-mannosidase activity to include β-N-acetylmannosaminidase activity is based on an analogy to the α-N-acetylgalactosaminidase from Aspergillus niger, which is a very close homolog of α-galactosidase from the...
same fungus, differing only in 7 amino acids (Kulik et al. 2010).

In fact, α-N-acetylgalactosaminidase cleaves aminosugars by the same mechanism as α-galactosidase. This differs completely from the β-N-acetylhexosaminidase mechanism, which uses an oxazoline intermediate (Knapp et al. 1996). An analogous mechanism is expected in the proposed β-N-acetylmannosaminidase to β-mannosidase and that this transformation would therefore enable it to perform the former activity (expansion/modulation of the binding site for the 2-OH group to accommodate an NHAc moiety).

Results

Searching for β-N-acetylmannosaminidase activity in natural sources

We have screened a large variety of biological sources, e.g. eukaryotic and prokaryotic microorganisms, invertebrates, plant and mammal tissues for the β-N-acetylmannosaminidase activity using p-nitrophenyl-β-N-acetylmannopyranoside as a chromogenic substrate (Krenek et al. 2012).

Extracellular β-N-acetylmannosaminidase activity was searched for in a series of filamentous fungi (Abortiporus biennis 521 CCBAS, Bjerkandera adusta 930 CCBAS, Dichomitus squalens 750 CCBAS, Phanerochaete chrysosporium 571 CCBAS, Pleurotus ostreatus 477 CCBAS, P. ostreatus 741 CCBAS, Trametes hirsuta 610 CCBAS, Trametes versicolor 612 CCBAS, T. versicolor 143 CCBAS, Trametes pubescens 89 CCBAS and Trametes villosa 678,8 CBS). Both intra- and extracellular β-N-acetylmannosaminidase activities were searched for in bacteria cultivated under various conditions (Bacillus—5 strains, Pseudomonas—5 strains, Rhodococcus—17 strains, Escherichia—3 strains, Enterobacter—2 strains, Nocardia and Streptococcus). Homogenates from a panel of invertebrates consisting of Ensifera (cricket), Chorthippus (grasshopper), Tenebrio molitor (mealworm), Acheta domesticus (house cricket) and Helix pomatia (snail) were screened for β-N-acetylmannosaminidase activity in three buffers of different pHs. The same set of buffers was used for rat liver extract and extracts from germinated seeds (Lens culinaris, Pisum sativum and Phaseolus vulgaris). Unfortunately, no β-N-acetylmannosaminidase activity was detected in the tested material.

Sugar docking into the native enzyme

As searching for new β-N-acetylmannosaminidase in native sources was unsuccessful, we decided to perform a mutagenesis of the recombinant β-mannosidase with the aim of modifying its ability to accommodate the N-acetyl group of ManNAc and to catalyze the hydrolysis of N-acetyl-β-D-mannopyranosides. Two structures of β-mannosidase from B. thetaiotaomicron (PDB IDs: 2JE8 and 2WBK) were examined. 2JE8 (Figure 1A) is a native protein complexed with 2-[3-(2-hydroxy-1,1-dihydroxymethyl-ethylamino)-propylamino]-2-hydroxymethyl-propane-1,3-diol (Tailford et al. 2007), while 2WBK corresponds to its mutant (E555Q) with 2,4-dinitrophenyl-2-deoxy-2-fluoro-β-D-mannopyranoside representing a potential

Fig. 1. (A) X-ray structure of β-mannosidase from B. thetaiotaomicron (PDB ID: 2JE8, chain A) shown in secondary structure representation (helixes—red, β-sheet—yellow; random coil—green) complexed with 2-[3-(2-hydroxy-1,1-dihydroxymethyl-ethylamino)-propylamino]-2-hydroxymethyl-propane-1,3-diol in active site of enzyme (in blue circle). (B) Superimposition of active sites of native enzyme (2JE8) in blue and its mutant (2WBK) in yellow, catalytic residues are highlighted in bold. (C) Superimposition of active sites of chains A (blue) and B (red) for structure PDB ID: 2JE8, catalytic residues are in bold. Color version of the figure is available Online.
Protein engineering of β-mannosidase

Michaelis–Menten complex (Offen et al. 2009). Amino acids interacting with the saccharide are exactly the same except for Glu555 (2JE8)/Gln555 (2WBK). We superimposed all binding sites of both structures (Figure 1B; root-mean-square deviation, RMSD = 0.45 Å) and also binding sites in chains A and B in 2JE8 (Figure 1C; RMSD_{A→B} = 0.08 Å), as the enzyme crystallized as a dimer in the unit cell. The superimposition showed almost no difference, therefore the docking and mutagenesis study was only conducted on the chain A binding site of the native enzyme. The accuracy of the docking algorithm was verified by cross-docking p-nitrophenyl β-mannopyranoside (pNP-β-Man) in S_{2} skew-boat conformation into the A chain of the native protein and comparing with the crystal structure of 2WBK (the E555Q mutant) with bound 2,4-dinitrophenyl-2-fluoro-β-p-mannopyranoside in S_{2} skew-boat conformation (Offen et al. 2009) (Supplementary data, Figure S1). The RMSD of 1.48 Å between docked p-nitrophenyl-β-o-mannopyranoside and 2,4-dinitrophenyl-2-fluoro-β-o-mannopyranoside in the crystal structure indicates a difference between the crystal and docked saccharide, which is mainly caused by the orientation of the p-nitrophenyl and o-p-nitrophenyl rings, respectively. The difference in the torsion angle C_{2α}−O_{2}−C_{3}−C_{5}/glycosidic linkage is ≈ 30°. Focusing only on non-reducing saccharide, the slightly counter-clockwise position of the OH-groups of mannoside in the docked structure vs. 2-fluoro derivative of the mannoside in the crystal structure of the mutant leads to the difference in an RMSD of 0.78 Å. This could be explained by a different orientation of O6 resulting in different hydrogen bonding in the binding site (Supplementary data, Figure S1B). The different functional group on C2 of the mannoside could also influence exact positioning of the mannoside ring. Because the orientation of both sugar rings is similar, it can be stated that the S_{2} skew-boat conformation of the β-o-mannopyranoside used for docking gave an almost identical position to the experimentally determined 2-fluoro derivative.

Four ligands, including β-Man, β-ManNAc, pNP-β-Man and p-nitrophenyl β-D-N-acetylmannopyranoside (pNP-β-ManNAc), in three different conformations covering the 4C1→4H5→1S5 pathway (Figure 2) (Stoddart 1971) were docked into native β-mannosidase. Binding energies, as well as the relative binding energies for the chosen substrates calculated by the docking program AutoDock 3 in the most populated clusters are summarized in Table I.

For monosaccharides, the docking binding energies of β-Man and β-ManNAc were very similar, which was mainly because of the AutoDock 3 algorithm that could not cover the

![Fig. 2. Stoddart diagram for hydrolysis of glycosidic linkage with itinerary S_{2}→B_{2}→S_{2} for β-o-mannopyranoside (indicated by red arrow) with highlighted transition state B_{2} (red) and chosen itinerary of conformational states for β-o-mannopyranoside preceding Michaelis–Menten complex (indicated by blue arrow). Color version of the figure is available Online.](https://academic.oup.com/glycob/article-lookup/doi/10.1093/glycob/cvab010)

### Table I. Docking and free energies (kcal/mol) of different β-mannosidase/saccharide complexes in most populated clusters predicted from docking calculations

<table>
<thead>
<tr>
<th>Saccharide</th>
<th>Conformation</th>
<th>Wild type</th>
<th>Mutant (E462DE555D)</th>
<th>Mutant (W395DE555D)</th>
<th>Mutant (W395DE462D)</th>
<th>Mutant (W395DN461S)</th>
<th>Mutant (N178AD199S)</th>
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<tr>
<td>β-o-Man</td>
<td>4C_{1}</td>
<td>−9.45</td>
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<td>−9.41</td>
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<td>−10.00</td>
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<td>−11.33</td>
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<td>p-NP-β-o-ManNAc</td>
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<td>−10.80</td>
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</table>

*p*-saccharides were modeled in 4C_{1}, 4H_{5} and 1S_{5} configuration.
small difference in the functional group of these two substrates at the C2 position. This is a general problem in protein (enzyme)/carbohydrate interactions, as differences in calculated binding energies are usually very close to each other. Because of the general similarities of sugar rings, all docking software usually places any sugar ligand into or very close to the binding site of the protein (enzyme). This always results in a favorable binding score, as several hydroxyls can participate in the interaction. As a result, energy differences of \(<2\) kcal/mol will not significantly distinguish binders and non-binders (Morris et al. 1998; Mishra et al. 2012). However, the docking study was also used to find the proper configuration and orientation of the substrates in the active site of the enzyme, where the position of the glycosidic linkage (above the catalytic residues Glu462 and Glu555) and the stabilization through side chains of the active site residues (Asn178, Trp198, Asp199, Trp200, Trp395, Cys424, Asn461, Tyr537, Trp395 and Trp645) (Tailford et al. 2007) needed to be considered.

Analysis of the binding modes of docked pNP-\(\beta\)-Man with the \(4C1\) conformation was used for both saccharide units. To examine the behavior and rearrangement of the binding site of the \(\beta\)-mannosidase and the conformation of the saccharide, we decided to work further with monosaccharides and disaccharide unit in the active site. For the above reason, we used to equilibrate the system) in explicit solvent without and with \(\beta\)-D-mannopyranosyl and \(\beta\)-D-mannopyranosyl-\(\beta\)-(1→4)-D-mannopyranosyl (using all saccharides in the \(4C1\) conformation, overview of the simulations performed is summarized in Supplementary data, Table S1). The majority of our discussion will focus on the analysis of the binding site topography and the position of the saccharide in the active site after the equilibration of the system.

To quantify the rearrangement of the system, the RMSDs of \(C_{\alpha}\) atoms between the simulated and experimental structure were computed as a function of time. Each simulation showed a rise of \(\sim 0.60\) Å relative to the starting structure (Figure 4A). After 2 ns, no further RMSD development was seen. All complexes converged to RMSDs of \(\sim 1.2\) Å, except for the overall value of the \(C_{\alpha}\) RMSD for the \(\beta\)-mannosidase/\(4C1\) \(\beta\)-Man that had a slightly higher variance (by \(0.25\) Å) than the others. Some deviation from the crystal structure is always expected in MD simulations, often due to the fact that the crystal structure contains interdomain interactions that are generally absent in the MD simulations.

To analyze the topography of the binding site, RMSDs were also calculated only for \(C_{\alpha}\) (Figure 4B) and all atoms (Figure 4C) of the residues which are involved in the saccharide binding (Asn178, Trp198, Asp199, Trp200, Trp395, Cys424, Asn461, Tyr537, Trp395 and Trp645). The geometry of the binding site in free \(\beta\)-mannosidase and its complexed state with dimannoside was equilibrated to a similar topography with respect to the starting conformation. The largest differences were seen for \(\beta\)-mannosidase/\(\beta\)-Man, giving a less effective rearrangement of the binding site. The detailed analysis revealed a movement of Asn178 out from the binding site of the enzyme, which is not at an effective distance (polar interaction) to interact with the saccharide in the binding site. The distance between Asn178 (N-atom of amide) and Asp199 (O-atom of carboxyl) lengthened from 2.8 to 14.5 Å (Figure 4D and E). This may indicate that Asn178 is not responsible for the binding of \(\beta\)-D-mannopyranosyl. The flexibility of Asn178 is enhanced by its position within a flexible loop.

MD simulation on the native enzyme

To examine the behavior and rearrangement of the binding site of \(\beta\)-mannosidase and the configuration of the saccharide, we performed multiple MD simulations (3 \(\times\) 5 ns – timescale used to equilibrate the system) in explicit solvent without and with \(\beta\)-D-mannopyranosyl and \(\beta\)-D-mannopyranosyl-\(\beta\)-(1→4)-D-mannopyranosyl (using all saccharides in the \(4C1\) conformation, overview of the simulations performed is summarized in Supplementary data, Table S1). The majority of our discussion will focus on the analysis of the binding site topography and the position of the saccharide in the active site after the equilibration of the system.

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**In silico mutagenesis of \(\beta\)-mannosidase and the docking into the mutated enzyme**

In silico mutation was performed on residues Asn178, Asp199, Trp395, Cys424, Asn461, Glu462 and Glu555. Overall, 47 mutants were generated as a combination of single to triple-point mutants. In silico mutants were generated by TRITON, which uses MODELLER software to model the mutant

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**Fig. 3.** Active site (blue) of \(\beta\)-mannosidase with docked \(p\)-nitrophenyl-\(\beta\)-D-mannopyranoside (cyan) in \(4C1\) (A) chair, \(4H_3\) twist (B) and \(1S_5\) skew-boat (C) conformations. Color version of the figure is available Online.
structures using homology modelling. Due to the fact that single- and triple-point mutants exhibited high diversity in docking and MD equilibration studies, only the results of the double-point mutants for docking and MD will be presented here unless otherwise stated.

The mutations considered were Asn178 to Ala or Ser, Asp199 to Ala or Ser, Trp395 to Asp or Asn, Asn461 to Ala or Ser, Cys424 to Ala or Ser, Glu462 to Asp and Glu555 to Asp taking into account the formation of the oxazoline intermediate for the hydrolysis (i.e. the replacement of Glu with Asp), the conformational itinerary of β-mannosidase (i.e. $^1S_4$ conformation, Trp to Asp and Asn to Ser) or the alanine–serine–alanine–proline (ASAP) stabilization of the acetamido group (i.e., Trp to Asp, Asn to Ser or Ala and Asp to Ser). Finally, five double-point mutants were prepared in silico, E462DE555D, W395DE555D, W395DE462D, W395DN461S and N178AD199S.

The binding energies obtained by the docking (Table I) for these mutants did not show any dependence of an increase/decrease of the energy for the chosen saccharide conformation. With pNP-β-ManNAc, ligands in the $^4C_1$ conformation were
positioned differently than with the native β-mannosidase in the E462DE555D (Figure 5) and W395DE462D mutants (Supplementary data, Figure S2). In contrast to the native enzyme, where the p-nitrophenyl group pointed out of the binding site, it interacted with active site amino acid residues and contributed to the binding energies from the docking studies. The $^{1}S_{2}$ conformation of the sugar was docked much deeper into the active site of both mutants and the glycosidic linkage and acetamido group were well oriented to the catalytic residues of the enzyme. The docking modes of pNP-β-ManNAc with the W395DN461S, W395DE555D and N178AD199S mutants (Supplementary data Figures S3–S5) were similar to the native β-mannosidase. W395DN461S and W395DE555D formed additional polar interactions with the acetamido group of pNP-β-ManNAc in the $^{1}S_{2}$ conformation. With N178AD199S, the $^{4}C_{1}$ conformation of pNP-β-ManNAc was docked deeper into the active site of the enzyme compared with the $^{1}S_{2}$ conformation. The glycosidic linkage and acetamido group were well oriented and stabilized by additional polar interactions.

**MD simulations on mutated enzymes**

To examine the behavior and rearrangement of the mutated binding site of β-mannosidase mutants and the configuration of the saccharide, we performed 5 ns MD simulations in explicit solvent with β-ManNAc in the $^{4}C_{1}$ and $^{1}S_{2}$ conformations (Supplementary data, Table SII). All simulations were designed under conditions described in the Materials and methods. The analysis of the simulation was focused on the binding site topology and the position of the saccharide in the active site after the equilibration of the system.

To qualitatively analyze their conformational stability, the $C_{α}$ atom RMSDs were computed as a function of time. Each simulation gave a rise of $\sim$0.70 Å (Figure 6A) relative to the starting structure and after 3 ns no further RMSD development is seen. All the β-mannosidase mutant complexes converged at RMSDs of $\sim$1.4 Å.

To analyze the topology of the binding site, the RMSDs were also calculated for all residues involved in the saccharide binding (Figure 6B) and between C1 of β-ManNAc and carboxylic oxygens OE1 of both catalytic residues E/D462 (Figure 6C) and E/D555 (Figure 6D).

The RMSDs of the binding sites in E462DE555D with β-ManNAc ($^{1}S_{2}$ conformation; Figure 7A) and N178AD199S ($^{4}C_{1}$ conformation; Supplementary data, Figure S6) equilibrated to a close value of $\sim$1.2–1.4 Å, similar to the native enzyme (Figure 6B). The shortening of catalytic residues (Glu for Asp) in E462DE555D lead to significant changes in the distances between their carboxylic oxygens and the C1 of the sugar, demonstrating a higher flexibility of the binding site and the freedom of the mannoside to rotate (Figure 7A). The distance fluctuated between 5.9 and 13.5 Å for D462 (Figure 6C), and 4.2 and 9.7 Å for D555 (Figure 6D).

The N178AD199S mutant showed that replacing N178 with alanine made the mutated residue more involved in the enzyme saccharide complex interaction compared with the native enzyme, where N178 exhibited a high flexibility. The starting distance and the distance at the end of the simulation from C1 of β-ManNAc was 5.9 and 6.8 Å for E462 (Figure 6C), and 5.3 and 5.7 Å for E555, respectively (Figure 6D). Together with the second mutation, this may indicate that the ASAP stabilization on the acetamido group takes place in the mutated active site of the enzyme.

Mutations affecting the aromatic residue Trp395 resulted in significantly higher RMSDs, demonstrating a less effective rearrangement of the binding site (Figure 6B) to accommodate the substrate. In all cases, the binding sites showed a drift of 1–2 Å at 3 ns, which was the most significant change in RMSD for all the abovementioned MD simulations. Distance between the C1-carbon of β-ManNAc and the OE1-oxygen of the carbonyl D462 and E555 varied between 3.6–9.9 and 5.4–8.2 Å for the W395DE462D mutant (Figure 6C and D). Similar results were obtained for W395DE555D (data not shown). In the W395DN461S mutant, these differences were even more distinct with values of 6.0–12.1, and 4.1–8.1 Å for D462 and E555, respectively. The structures of the mutants at the beginning and at the end of simulations are depicted in Figure 7B (for W395DE462D) and Supplementary data, Figure S7 (for W395DN461S). The trajectory analysis indicated that the mutation of W395 (even if a non-stacking residue) disturbs the active site of the enzyme and the presence of β-ManNAc cannot effectively rearrange the active site to tightly bind the substrate.

In silico mutagenesis proposed several residues whose replacement could change the specificity of the enzyme. More than 30 mutants were prepared in vitro (one or more amino acids changed) and their abilities to cleave β-ManNAc-containing compounds were tested directly in crude bacterial extracts. However, no β-N-acetylmannosidase activity has been detected. In spite of this, the relative β-mannosidase activity in tested homogenates decreased to 17% and lower (Supplementary

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**Fig. 5.** Active site (blue) of mutant enzyme E462DE555D (mutated residues in yellow) with docked p-nitrophenyl-N-acetyl-β-D-mannopyranosamide in $^{4}C_{1}$ chair (A), $^{4}H_{2}$ twist (B) and $^{1}S_{2}$ skew-boat (C) conformations. Color version of the figure is available Online.
Discussion

The screen of a large variety of biological sources, e.g. eukaryotic and prokaryotic microorganisms, invertebrates, mammal tissues, did not detect β-N-acetylmannosaminidase activity in the native sources and to date, no GH cleaving β-N-acetylmannopyranosamine residues, e.g. β-N-acetylmannopyranosaminidase, have been identified. Therefore, we decided to perform protein engineering on the recombinant β-mannosidase to accommodate the N-acetyl group of ManNAc and to catalyze the hydrolysis of β-N-acetyl-D-mannopyranosamine. The structure of β-mannosidase (Offen et al. 2009) and metadynamics studies (Ardevol et al. 2010) strongly supported the electrophilic migration of the anomeric carbon along the 1S5→B2,5→OS2 itinerary. Experimental support for catalysis along trajectories passing through boat conformations indicated the 1S5 skew-boat conformation for the Michaelis complex, coupled to the OS2 skew-boat conformation for the intermediate. This Michaelis complex displays a classical geometry for nucleophilic substitution to the anomeric carbon. The 1S5 skew-boat conformation is reported to be the last conformation before the hydrolysis of the glycosidic linkage (Stoddart 1971; Ardevol et al. 2010).

Docking and MD simulation of native β-mannosidase

To also cover conformational changes of the saccharide preceding hydrolysis of the glycosidic linkage as well, we also considered the 4C1→H5→S2 itinerary for both β-D-mannopyranose and p-nitrophenyl-β-D-mannopyranoside. Based on the results of the semi-flexible docking, the active site identifies the best possible conformation of the saccharide (1S5 conformation) from the chosen itinerary in the terms of metadynamics and X-ray studies. Analysis of the binding modes of docked p-nitrophenyl derivatives with the 4C1→H5→S2 itinerary exhibited the opposite behavior in terms of binding energies to those with β-D-mannopyranose. This indicates that the substrate needs a carrying compound (another saccharide unit or a p-nitrophenyl derivative), which can better mimic the functional property of the β-mannosidase (hydrolysis of the glycosidic linkage). MD (equilibration state) indicated that the Asn178 residue in the native enzyme does not participate in building up the enzyme-substrate complex due to the rearrangement of the active site.

Fig. 6. (A) RMSDs for Cα of whole simulated system calculated from initial structure and plotted as a function of time. (B) RMSDs from initial structure calculated for all atoms of binding site residues. (C) Change in distance between C1-carbon of β-ManNAc and OE1-oxygen of carboxyl D462 (or OE1-oxygen of carboxyl E462) or (D) OE1-oxygen of carboxyl D555 (otherwise OE1-oxygen of carboxyl E555) in MD simulation. Color version of the figure is available Online.
Docking and MD simulation of mutated β-mannosidase

Generally, the in silico mutagenesis study of β-mannosidase using docking is not expected to provide correct quantitative scores for all mutants, but merely to identify "hot spot" amino acids where mutations are likely to markedly affect binding affinity. Residues were mutated either to consider the potential stabilization of the acetamido group of β-ManNac (Letts et al. 2005), the conformational itinerary of β-mannosidase (Tailford et al. 2008; Offen et al. 2009; Ardevol et al. 2010) or the participation of the acetamido group in the creation of the oxazoline intermediate (Knapp et al. 1996; Mark et al. 2001). The stabilization of the acetamido group of β-ManNac was based on a comparison of α-N-acetylgalactosaminidases and α-galactosidases, and/or α-N-acetylgalactosetransferases and α-galactosetransferases. Also a highly conserved amino acid sequence ASAP responsible for the stabilization of the acetamido group was discovered (Letts et al. 2005). Concerning participation of the acetamido group in forming the oxazoline intermediate, in N-acetylhexitosaminidases it was shown that one of the two catalytic acidic amino acid residues is shorter (aspartic acid) than the other (glutamic acid). It helps the acetamido group to make a stable oxazoline intermediate as a key preceding step of the glycosidic bond hydrolysis (Knapp et al. 1996; Mark et al. 2001).

The in silico mutagenesis combined with the docking studies of the mutant enzyme showed that double-point mutants could be a suitable way to extend the β-mannosidase activity to β-N-acetylmannosaminidase activity, while the single- and triple-point mutations gave a high diversity for docking and MD studies. Further analyses of double-point mutants with MD approaches were done to get a better understanding of the configuration of the saccharide during the simulations. The selected double-point mutants indicated a possible ASAP stabilization of the acetamido group (the Asn178 to alanine mutation) or the creation of an oxazoline intermediate by shortening the catalytic residues from Glu to Asp. Mutations of the W395 residue led to a largely disturbed enzyme active site that prevented β-ManNac being bound in a tight enzyme–substrate complex.

The in vitro prepared mutants did not show β-N-acetylmannosaminidase activities. However, the screening was done only in crude bacterial extracts. As some of the lysates also did not exhibit β-mannosidase activity, it could be caused by the low gene expression, badly folded protein or affecting a crucial amino acid for the binding site architecture or enzyme catalytic mechanism. Therefore, further optimization of the screening conditions should be done to ensure that activity is not under a detection limit of the currently used methodology. Quantum mechanics/molecular mechanics approaches should be employed.
to decipher the contributions of individual amino acid substitutions to the catalytic mechanism of the enzyme.

Materials and methods

Starting PDB structure for in silico studies

The structure of the β-mannosidase from *B. thetaiotaomicron* in a complex with 2-[3-(2-hydroxy-1,1-dihydroxymethyl-ethylamino)-propylamino]-2-hydroxymethyl-propane-1,3-diol (PDB ID: 2JE8) (Tailford et al. 2007) (Figure 1A) and 2,4-dinitrophenyl-2-deoxy-2-fluoro-β-D-mannopyranoside (PDB ID: 2WBK) (Offen et al. 2009) were taken from the Protein Data Bank (Berman et al. 2003). All water molecules, co-crystallized molecules and ligands were removed from the initial PDB structures. The hydrogen atoms were added using the software WHAT IF (Friend 1990). Kollman united atom charges were used for the protein partial atom charges (Weiner et al. 1984). Non-polar hydrogen atoms were removed and their charges were merged into the bonded heavy atom using the in-house developed software TRITON (Prokop et al. 2008). Solvation parameters were also assigned with TRITON.

In silico ligand set preparation

The structures of the ligands were modeled using either Glycam biomolecule builder (Woods Group, 2005–2011) or Avogadro (open source, http://avogadro.openmolecules.net/). The ligands used for docking computations were chosen on the basis of a previous theoretical and experimental study of β-D-mannosidase–ligand interaction (Tailford et al. 2008; Offen et al. 2009; Ardevol et al. 2010). Initial structures of the ligands were geometry optimized by the Hartree–Fock method with the 6-31G* basis set, and the electrostatic potential was calculated by the same method using the program GAUSSIAN 03 (Frisch et al. 2004). The charge fitting procedure was done using the restrained electrostatic potential method using the antechamber (Wang et al. 2006) module of the package AMBER 11 (Case et al. 2010). The output mol2 file was converted into pdbq format, which was used as a valid input for the AutoDock (Morris et al. 1998) ligand preparation procedure. Non-polar hydrogen atoms of the ligand were removed and their charges were merged into the bonded heavy atom using TRITON (Prokop et al. 2008). During docking, all exocyclic groups were set as rotatable, except for the amido group of β-ManNAc and NO₂ group of the p-nitrophenyl derivative.

Docking

Docking was performed using the software AutoDock 3.0 (Morris et al. 1998) under the inhouse developed software TRITON (Prokop et al. 2008). The Lamarckian genetic algorithm was used to search for energetically favored binding modes. The grid box was set to cover the active site with a 0.375 Å spacing. The number of runs of the Lamarckian genetic algorithm was set to 100 and 5,000,000 energy evaluations were used per run. The population size was 50 individuals. The evaluation of ligand conformations using cluster analysis with a 0.5 Å RMS tolerance was performed with AutoDock. The docking output *dlg* file was visualized with TRITON software and individual clusters were ranked according to their docked energy.

Preparation of in silico mutants

The 3D structures of the in silico mutants were prepared by homology modeling, using the software TRITON, which uses MODELLER (http://salilab.org/modeller/modeller.html) software for modeling the mutant structure. Hydrogen atoms in the receptor structures were added using AutoDockTools (Morris et al. 1998). In silico mutation of the amino acid residues was applied and a total of 47 in silico mutants of β-mannosidase were obtained. When modeling each mutant one by one, MODELLER optimizes the complete structure of the mutant followed by a short MD simulation to equilibrate the whole protein geometry.

Molecular dynamics

MD simulations were performed using the *sander* module from AMBER 11 (Case et al. 2010). Input coordinates were taken from the structure of the β-mannosidase complex with 2-[3-(2-hydroxy-1,1-dihydroxymethyl-ethylamino)-propylamino]-2-hydroxymethyl-propane-1,3-diol (PDB ID: 2JE8). Structures were built up with the *xLeaP* module of AMBER. The AMBER force field parm99sb (Wang et al. 2000) was used for the simulation of amino acids and parameters for monosaccharides were taken from the GLYCAM06 force field (Kirschner et al. 2008). The standard AMBER scaling factors, i.e. SCEE and SCNB, were implicitly defined by AMBER11 to be 1. Hydrogens were added with *xLeaP*. The system was explicitly solvated with TIP3P water molecules using Solvate software (Grubmuller 1996). The minimum distance of the protein atoms from the walls of the truncated octahedron water box was set to 15 Å. In order to maintain an overall zero charge for the system, 11 Na⁺ ions were added with the *xLeaP* module. Before the production phase of MD simulation was run, a stepwise equilibration of the system was performed. First, water molecules and ions were energy minimized and consequently heated from 5 to 298.15 K (30 ps). To complete the equilibration of the solvent, a 100-ps MD simulation of ions and water molecules was performed at 298.15 K. The equilibration continued with energy minimization of the whole system and a gradual decrease in the restraints that were imposed on the protein and saccharide molecules. After elimination of the restraints, a 160-ps MD simulation with slow heating of the system from 5 to 298.15 K was performed, followed by 50 ps of dynamics at 298.15 K. Both MD simulations were carried out under a constant pressure of 1 atm. A 5 ns production phase of MD simulations were run at a constant pressure of 1 atm and temperature of 300 K. The equations of motion were integrated with a time step of 2 fs and system coordinates were saved each 1 ps. The SHAKE (Ryckaert et al. 1977) algorithm was applied to constrain bonds with hydrogen atoms. Electrostatic interactions were calculated by using the Particle Mesh Ewald (Darden et al. 1993) method with a cutoff of 9 Å. MD trajectories were analyzed using the *praj* module from AMBER 11 and visualized with the software VMD (Humphrey et al. 1996).

Enzyme assay

Enzyme activities were determined using p-nitrophenyl glycosides as substrates. p-Nitrophenyl α-D-mannopyranoside, pNP-β-Man and p-nitrophenyl β-D-acetylglucopyranoside (pNP-β-GlcNAc) were purchased from Sigma-Aldrich. pNP-β-ManNAc was prepared as described previously (Kfenek et al. 2012). The
reaction mixture contained 10 µL of 10 mM pNP-sugar, 10 µL of 50 mM citrate–phosphate buffer, pH 3.5, and 30 µL of the enzyme solution (lysate). The reaction was incubated at 35°C for 10 min and stopped by the addition of 1 mL of 0.1 M Na2CO3. The p-nitrophenol released was determined spectrophotometrically at 420 nm. One unit of enzyme activity is defined as the amount of enzyme releasing 1.0 µmol of p-nitrophenol per minute under the assay conditions. Fifty millimolars of citrate–phosphate buffer, pH 3.0, 50 mM citrate–phosphate buffer, pH 5.0, and 50 mM Tris–HCl (pH 7.0) were used for pNP-β-ManNAc activity screening in native sources.

Searching for β-N-acetylmannosaminidase activity in natural sources

The tested strains of fungi were obtained from the Culture Collection of Basidiomycetes (Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague) and from the Centraalbureau voor Schimmelcultures (Netherlands). Bacterial strains were from the Czech Collection of Microorganisms (Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague) and the National Collection of Industrial, Food and Marine Bacteria, UK. Rat liver was kindly provided by Palacký University in Olomouc, Czech Republic, invertebrates and plant seeds were purchased from the local shops. The sample preparations for screening procedures are described in detail in the Supplementary data.

DNA manipulations

A synthetic gene of the β-mannosidase gene from B. thetaiotaomicron (UniProt ID Q8AAK6) (GeneArt, Life Technologies) was inserted into the NdeI and HindIII sites in the pET30a plasmid (Novagen). The codon frequency was optimized using GeneArt’s own software for production in E. coli. The set of mutants was prepared using the primers shown in Supplementary data, Table SIII. The prepared vectors were transformed into E. coli BL21pLys (DE3) (Agilent Technologies—Stratagene Products). E. coli cells bearing corresponding plasmids were grown in 50 mL Falcon tubes with 10 mL of LB broth and 50 µg kanamycin mL−1. After 3.5 h of cultivation at 37°C, the expression of β-mannosidase or its mutants was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested after 24 h, centrifuged, the cell pellet was resuspended in a lysis buffer composed of 0.004 M MgCl2, 0.05 M NaCl, lysozyme (1 g/L−1), PMSF (10 mL/L−1) and Triton X-100 (0.5%) and incubated at room temperature and 220 rpm for 30 min. The mixture was sonicated (for 2 min three times), centrifuged and the lysate was assayed for β-mannosidase/β-N-acetylmannosaminidase activity.

Authors’ contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supplementary Data

Supplementary data for this article are available online at http://glycob.oxfordjournals.org/.

Conflict of interest statement

None declared.

Funding

The access to computing and storage facilities owned by parties and projects contributing to the National Grid Infrastructure MetaCentrum provided under the program “Projects of Large Infrastructure for Research, Development, and Innovations” (LM2010005) is highly appreciated. This work is supported by the Czech Science Foundation (P207/10/0321) and EU project NOVOSIDES FP7-KBBE-2010-4-265854 (MSMT 7E11101). This work was further supported by the project “CEITEC—Central European Institute of Technology” (CZ.1.05/1.1.00/02.0068) from European Regional Development Fund and the Institute of Microbiology research concept RVO6138971.

Acknowledgments

Authors thank Dr Peter Kulhánek (Masaryk University, Czech Republic) for valuable discussions.

Abbreviations


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