Structural Basis of Outstanding Multivalent Effects in Jack Bean α-Mannosidase Inhibition

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Abstract: Multivalent design of glycosidase inhibitors is a promising strategy for the treatment of diseases involving enzymatic hydrolysis of glycosidic bonds in carbohydrates. An essential prerequisite for successful applications is the atomic-level understanding of how outstanding binding enhancement occurs with multivalent inhibitors. Herein we report the first high-resolution crystal structures of the Jack bean α-mannosidase (JBα-mann) in apo and inhibited states. The three-dimensional structure of JBα-mann in complex with the multimeric cyclopeptoid-based inhibitor displaying the largest binding enhancements reported so far provides decisive insight into the molecular mechanisms underlying multivalent effects in glycosidase inhibition.

Modulation of the multiple biological activities of glycosidases is a major goal for drug discovery.[1] Catalytic hydrolysis of glycosidic linkages, the most stable covalent single bonds within biopolymers, is a fundamental process involved in key cellular events, including energy uptake and post-translational modifications of glycoproteins.[2] The multivalent design of glycosidase inhibitors has recently experienced rapid development with the disclosure of glycomimetic clusters showing outstanding affinity enhancement over the corresponding monovalent ligands (up to five orders of magnitude).[5–7] Although intensive efforts have been made to rationalize the inhibitory multivalent effect observed, how multimeric inhibitors and glycosidases interact at the atomic level remains unknown.[6] Recent studies in the field have logically focused on Jack bean α-mannosidase (JBα-mann), since this high-molecular-weight (220 kDa) zinc enzyme,[8] is the most sensitive to multivalent binding known to date.[6]

JBα-mann is a member of the retaining glycoside hydrolase family 38 (GH38), which contains therapeutically relevant α-mannosidases.[8a,b] These mammalian enzymes participate in the biosynthesis and catabolism of N-glycans in cells[8a] and as such constitute targets for the treatment of cancers and lysosomal diseases.[9] In the absence of a 3D crystallographic structure for JBα-mann, interactions with multivalent inhibitors have been studied by indirect methods, such as atomic force microscopy, dynamic light scattering, NMR spectroscopy, or mass spectrometry.[5–7] These studies have led to a number of competing hypotheses and binding models involving large aggregates, additional interactions with enzyme subsites, or the formation of discrete cross-linked complexes.[5–7] Herein we report the first high-resolution crystal structures of apo JBα-mann and its complex with the 36-valent cluster Ι[8] (Figure 1). The experimental observation of multivalent interactions in association with molecular modeling suggests a clear rational basis for the outstanding affinity enhancement observed.

The apo structure of JBα-mann was solved by X-ray protein crystallography at 1.84 Å resolution (Protein Data Bank, PDB entry 6B9O). Molecular replacement with bovine lysosomal α-mannosidase (PDB entry 1OTD)[9] was successfully used for the initial structure determination. This protein was chosen following sequence alignment of the published sequence for JBα-mann[8a] against the full PDB content, which gave 40% identity (see Tables S1 and Figure S1 in the Supporting Information). The crystallographic asymmetric unit has one JBα-mann protein composed of two LH heterodimers, each formed by two distinct chains, L1 and H1 (L: light chain and H: heavy chain; Figure 2). Figure 2 also shows the symmetry-related (LH) complex (LH1a and LH2a). The four active sites, identified by the zinc ions in the H-chain, are turned toward a cavity at the center of the complex composed by the four LH heterodimers. We anticipated that this large pocket could accommodate large multivalent inhibitors, such as Ι.

How JBα-mann binds multimeric inhibitors is key to our analysis of the outstanding multivalent effects observed. To gain detailed insight at the atomic level, we attempted to determine the structures of complexes of JBα-mann with diverse cyclopeptoid-based iminosugar clusters.[7] Extensive
Cocrystallization of enzyme:inhibitor complexes was carried out by a Mosquito robot using a sitting-drop vapor-diffusion method. Remarkably, well-diffracting crystals were obtained with 1, which displays the largest binding enhancement observed so far for a glycosidase (Figure 1). The structure of the complex of JBα-man with the 36-valent cluster 1 was solved by X-ray protein crystallography at 2.0 Å resolution (PDB entry 6B9P). The overall structure of this complex was similar to that of the apo form of JBα-man with the same crystal parameters (space group and cell dimensions), thus indicating that multivalent inhibitor binding does not perturb the protein. The X-ray complex structure showed the mode of binding of four iminosugar heads of the 36-valent cluster 1 in the catalytic site of each LH heterodimer. In the X-ray structure, only one iminosugar head was clearly visible for each LH heterodimer in the electron-density map at 2.0 Å resolution, suggesting the absence of a secondary binding site (Figure 3; see also Figure SI2). Nonspecific interactions at the surface of the enzyme can, however, not be excluded. The iminosugar head of the multivalent inhibitor is buried in the catalytic pocket in the H-chain, whereas the aliphatic linker makes hydrophobic contacts with the protein surface (Figure 3; see also Figures SI2–SI6).

Figure 1. Mono- and 36-valent inhibitors of JBα-man. Pr = propyl.

Figure 2. Ribbon representation of two (LH)2 complexes, one formed by LH1 (L1 in cyan and H1 in green) and LH2 (in red) and a symmetry-related complex formed by LH1a (in yellow) and LH2a (in blue), and zinc atoms (gray spheres).

Figure 3. JBα-man protein structure in complex with an iminosugar head and aliphatic chain of 1; cyan: ribbon L-chain, green: ribbon H-chain, yellow sticks: carbon atoms, red sticks: oxygen atoms, blue sticks: nitrogen atoms, and gray sphere: zinc atom.

The zinc atom in the H-chain is clearly observed in an octahedral coordination environment with six atoms at distances between 2.1 and 2.3 Å (Figure 4): four oxygen atoms, two of them provided by protein aspartate residues (Asp145, Asp25) and two by the iminosugar head, and two nitrogen atoms provided by protein histidine residues (His23 and His386). The geometry and the intervening residues are the same as in previously reported structures from two other proteins of the same GH38 family (see Figure SI7), with the notable difference that the ligand in those previous structures provides only one zinc-coordinating oxygen atom. The residue Asp145 is characteristic of GH38 α-mannosidases and has been demonstrated as the catalytic nucleophile in JBα-man. On the basis of the expected distance (ca. 5.5 Å) between the two catalytic carboxylate groups in a retaining hydrolysis mechanism, Asp25 would seem the most likely candidate for the catalytic acid/base (see Figure SI6). Furthermore, the involvement of the two catalytic residues in the coordination of the zinc ion is believed to be essential to the mechanism of the catalytic glycosidic cleavage.

No electron density could be observed beyond the quaternary carbon branching point of the trivalent dendron of cluster 1. Since the considerable conformational flexibility of the cyclic peptoid core and associated ethylene glycol spacers precludes a priori direct information from X-ray crystallography, a 3D model of the full 36-valent cluster 1 was built and was successfully fitted into the cavity with the
program Coot[14] (see Figure SI8). The iminosugar heads were kept in the positions shown in the X-ray maps, the inhibitor core in the middle of the cavity, and the remaining part between the core and the iminosugar heads in an arbitrary conformation.

This encouraging result indicated that our binding model was sterically plausible. However, the absence of any signal corresponding to the disordered center of 1 in the electron-density map at 2.0 Å prompted us to develop a convenient method to obtain experimental evidence for its location. A combination of symmetry-lowering and low-resolution maps eventually enabled us to observe a signal corresponding to the inhibitor core. Since the cavity spans two asymmetric units in \( P_2_1_2_1_2 \), with one half of the cavity in each asymmetric unit, the disordered multivalent ligand might not respect the symmetry between both halves. To recover any diffraction signal, the whole cavity should be in a single asymmetric unit; therefore, the symmetry of both crystals (apo and holo) was reduced from orthorhombic (\( P_2_1_2_1_2 \)) to monoclinic (\( P_2_1 \)), thus expanding the asymmetric unit to include the two (LH)\(_2\) complexes. The corresponding structure factors were recalculated from the raw images to recover all the information present in the diffraction data. The corresponding models (apo and holo) were placed in the \( P_2_1 \) space group and re-refined, giving a very similar result to the \( P_2_2_2_1 \) refinement for the protein. Maps were calculated in this new space group. As no high-resolution details are expected owing to the flexibility of the multivalent ligand, map resolution was reduced from 2.0 to 5.0 Å, a suitable limit to enhance features of this size even with partial disorder. Electron-density maps for the protein were very similar (Figure 5). However, a signal was observed at the center of the cavity only for the data from the crystal with the multivalent ligand 1, thus strongly suggesting the presence of its core.

The conformational space of the multivalent ligand inside the cavity was explored by simulated annealing using the program Phenix,[15] starting from five different positions (generated manually with Coot[14]) for the linker arms and central core (see Figure SI9). In each of the five positions, the same four heads were kept in the corresponding catalytic pockets. The resulting five models clearly demonstrated that the four bound iminosugar heads belong to the same multivalent ligand. The observation of an electron-density signal at the center of the cavity provides experimental proof that this topology is the most probable (Figure 5). The present structural study confirms the formation of a 2:1 JB\(_{bt}\)-man:inhibitor sandwich-type complex, which was initially postulated on the basis of electron microscopy imaging, analytical ultracentrifugation measurements, and mass spectrometry experiments.[7]

The experimental observation of multivalent interactions in association with molecular modeling suggests a clear rational basis for the high affinity enhancements observed in JB\(_{bt}\)-man inhibition. In the binding topology proposed, the multivalent ligand occupies the cavity created by two (LH)\(_2\) complexes, with the core placed at the center of this cavity,

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**Figure 4.** Contacts of an iminosugar head with the protein and a zinc atom. Green sticks: protein, yellow sticks: 1, gray sphere: zinc atom, red spheres: water molecules. Left: full site; right: zoom on Zn coordination.

**Figure 5.** Electron-density low-resolution \( 2F_o-F_c \) map in blue (5 Å, 1.0 σ contour) with the protein represented in yellow, blue, green, and red: a) apo crystal; b) crystal with 1; c) close up on the density at the center superposed with the model of cluster 1 (cyan).
radial arms pointing towards the four catalytic sites present in the H subunits, and four iminosugar heads binding to them. The bridging of two (LH)₄ complexes by four iminosugar heads of the multimeric inhibitor results in a strong chelation effect, which explains the exceptionally large affinity enhancements observed.[7] The chelation effect is indeed considered to be the mode of binding underlying the most powerful multivalent effects reported to date.[16] Notably, inhibitor I fully occupies the cavity. Reduced multivalent effects may be anticipated for smaller clusters able to embrace only two catalytic sites simultaneously, or for giant clusters that would not fit inside the cavity, thus leading to other binding modes.[16,8,7] The iminosugars that are not buried in the catalytic pocket remain in close proximity, opening the possibility of multiple bind-and-recapture processes.[17]

In conclusion, the first high-resolution crystal structures of apo JBTα-man and of its complex with the 36-valent cluster I offer considerable insight at the atomic level into the way a glycosidease and a multimeric inhibitor interact to produce outstanding inhibitory multivalent effects. The X-ray crystallographic structure showed four ordered iminosugar heads of the 36-valent cluster I simultaneously engaging all four active sites of two JBTα-man molecules to form a strong sandwich chelate complex. The crystal structures presented herein provide the foundation for the rational design of multivalent inhibitors targeting JBTα-man and related clinically relevant GH38 α-mannosidases.

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Conflict of interest

The authors declare no conflict of interest.

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