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Computational design-of-experiment unveils the conformational reaction coordinate of GH125 α-mannosidases

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ABSTRACT: The conformational analysis of enzyme-catalysed mannoside hydrolysis has revealed two predominant conformational itineraries through B2,5 or \(^{3}H_4\) transition state conformations. A prominent unassigned catalytic itinerary is that of the exo-1,6-α-mannosidases belonging to CAZy family 125. A published complex of the Clostridium perfringens GH125 enzyme with a non-hydrolysable 1,6-α-thiomannoside substrate mimic bound across the active site revealed an undistorted \(^{2}C_1\) conformation and provided no insight into the catalytic pathway of this enzyme. Here we show, through a purely computational approach (QM/MM metadynamics) that sulfur-for-oxygen substitution in the glycosidic linkage fundamentally alters the energetically accessible conformational space of a thiomannoside when bound within the GH125 active site. Thus, while modelling of the conformational free energy landscape (FEL) of a thioglycoside strongly favors a mechanically uninformative \(^{3}C_1\) conformation within the GH125 enzyme active site, the FEL of the corresponding O-glycoside substrate reveals a preference for a Michaelis complex in an \(^{5}S_2\) conformation (consistent with catalysis through a B2,5 transition state). This prediction was tested experimentally by determination of the 3-D X-ray structure of the pseudo-Michaelis complex of an inactive (D220N) variant of the C. perfringens GH125 enzyme in complex with 1,6-α-mannobiose. This complex revealed unambiguous distortion of the –1 subsite mannoside to an \(^{0}S_2\) conformation, matching that predicted by theory, and supporting an \(^{0}S_2 \rightarrow B_{2,5} \rightarrow ^{1}S_5\) conformational itinerary for GH125 α-mannosidases. This work highlights the power of the QM/MM approach and identified potential shortcomings in the use of non-hydrolysable substrate analogues for conformational analysis of enzyme-bound species.

Figure 1. Mercator plot of major canonical conformations of a pyranose ring. The transition state conformations (boxed) and associated ground-state conformations of mannosidase conformational itineraries through transition states with \(B_{2,5}\) (blue) and \(^{3}H_4\) (green) conformations.

According to the principle of least nuclear motion, the conformations of the ground states of the enzymatic Michaelis complex, products, and (if relevant) associated intermediates must flank the transition states. While early analyses focused on the \(^{4}H_3\) transition state conformation, studies over the last 20 years have identified that all four major transition state conformations are co-opted by various enzymes working across the breadth of stereochemically-diverse carbohydrate substrates. As transition state mimicry provides a practical blueprint for the development of tight binding inhibitors, analysis of these reaction coordinates is proving invaluable in the design and application of transition-state mimicking species as mechanistic probes and therapeutic agents. The canvas upon which nature’s treasure-chest of glycosidases is depicted is the carbohydrate-active enzymes (CAZy) classifica-
mammnosides. A key challenge in synthesizing these compounds is understanding the conformational preferences of these enzymes. Studies on the conformational preferences of α- and β-mannosidases have been carried out using computational methods and experimental techniques. One approach involves the use of metadynamics simulations to explore the conformational space of the enzymes and to identify key residues that influence the catalytic process.

In a seminal study, Gregg and colleagues reported the creation of a thio-mannobiose substrate mimic spanning the active site of a GH125 enzyme (CpGH125) from Streptococcus pneumoniae. This mimic was used in conjunction with other computational methods to probe the conformational preferences of the enzyme. The star symbol plots the conformation observed in the thio-mannobiose substrate mimic. However, when the approach was applied to another GH125 enzyme (GH125 from Clostridium perfringens), the conformational space was found to be more restricted due to the presence of a C1-O5 bond, which was observed in the crystal structure of the enzyme.

When the QM/MM metadynamics approach was applied to the 'on-enzyme' complex of 1-thio-α-mannonypyranose and CpGH125, the free energy landscape was transformed such that the accessible conformational surface was dramatically restricted. This result, along with the experimental observations, supports the idea that the conformational preferences of the enzyme are crucial for its catalytic activity.

In conclusion, the use of computational methods in conjunction with experimental techniques provides valuable insights into the conformational preferences of α- and β-mannosidases. This knowledge is essential for the design of new inhibitors and for the development of therapeutic strategies.
The full conformational landscape of the –1 sugar ring of this competent substrate containing an O-glycosidic linkage was then calculated by QM/MM metadynamics, using the same procedure as in the case of the 1,6-α-thiomannobiose complex. Figure 2b shows that within this Michaelis complex (on-enzyme), an O-glycoside strongly favors an $O_2$ conformation, consistent with the α-mannosidase performing catalysis through an $O_2^->B_{2.5}$ $\rightarrow S$ conformational itinerary. QM/MM simulations of the reaction mechanism (Figures 3 and S2) starting from the $O_2^-$ conformation led to a $B_{2.5}$ transition state, in a dissociative reaction pathway generating a β-mannose product bound to CpGH125 with a $S_5/B_{2.5}$ conformation. Overall, this computational data, derived from the coordinate of the CpGH125 1,6-α-thiomannobiose complex, matches that proposed for GH families 2, 5, 26, 38, 76, 92, 113, and 130 (reviewed in Refs.1-6).

In order to validate, experimentally, the in silico prediction, an inactive variant in which the general acid (D220) of CpGH125 was mutated to a non-acidic asparagine residue was engineered. This catalytically-inactive variant was crystallized and soaked with the native O-glycosides, 1,6-α-mannobiose and -mannotriose to obtain pseudo-Michaelis complexes. Comparison of the structures of the ligand-free CpGH125 wildtype and ligand-bound D220N enzymes revealed no changes in the position of the amino acid side-chain or other residues, providing confidence that the observed ligand conformation was not a result of non-isomorphism. The CpGH125 D220N complexes, solved at resolutions of 2.10 and 1.55 Å (Supplementary Table 1), unambiguously reveal the –1 subsite mannoside distorted to a $O_2^-$ conformation (Figure 4a; for 1,6-α-mannotriose complex see Figure S3), matching that predicted a priori by computation.

The CpGH125 complexes highlight the molecular basis for catalysis, with a nucleophilic water poised for in-line nucleophilic attack at the anomeric carbon and with E393 positioned to act as the catalytic Brønsted base in an inverting mechanism, essentially as proposed previously.26 Interestingly, the nucleophilic water molecule is engaged in a hydrogen-bonding interaction with O3, rather than with O2, as was instead observed in the CpGH125 1,6-α-thiomannobiose complex. This interaction with O3 is reminiscent of that seen for the nucleophilic residue for a GH family 76 retaining 1,6-α-mannanase from Bacillus circulans,18 and is thus a feature of the non-metal dependent, family 76 and 125 α-mannosidases. Overlay of the CpGH125 D220N 1,6-α-mannobiose complex with the previously determined 1,6-α-thiomannobiose complex (Figure 4b) highlights the structural basis for the conformational differences; whilst the +1 (leaving group) subsite mannosides are essentially identical in terms of conformation and interactions, the –1 subsite mannoside moieties adopt different conformations, and match those predicted by computation. One major contributor to these different conformations is the longer C—S bond (1.89 vs. 1.48 Å for C—O); presumably as a result of this key structural difference the –1 thiomannoside in a $C_5^-$ conformation with an axial O2 group makes similar interactions to the pseudo-axial O3 of the mannoside in an $O_2^-$ conformation (Figure S4). Both the theory-based calculations and the subsequent experimental observation support a conformational itinerary for the inverting GH125 α-mannosidases that proceeds through a (near) $B_{2.5}$ transition-state conformation. This transition state is accessed following binding of the substrate in the ES complex in an $O_2^-$ conformation, Figure 3.

GH family 125 joins the growing list of mannos-enzyme pairs for X-ray crystallographic studies where they have provided mechanistically relevant insight into the conformations possible on enzyme, most notably in the case of distorted thioellipentaoside bound to Fusarium oxysporum cellulase of GH family 7.32 Could other thiglycoside complexes be misleading? In the case of an-

![Figure 3. Reaction coordinate for CpGH125 inverting 1,6-α-mannosidase obtained by QM/MM metadynamics with four collective variables. Hydrogen atoms have been omitted for clarity, except those of the carboxylate groups and water molecules.](image)

![Figure 4. (a) Observed electron density (2Fobs-Fcalc, $\sigma_A$ and maximum likelihood weighted) for the D220N 1,6-α-mannobiose complex of CpGH125, contoured at 0.31 electrons / Å$^3$. (b) Comparison of the C. perfringens GH125 complexes with 1,6-α-mannobiose (this work, brick red) with the 1,6-α-thiomannobiose complex (grey, PDB 3QT9, ref.26). D220 is the general acid; E393 is the general base; the proposed nucleophile water is shown.](image)
other inverting α-mannosidase, from GH family 47, a 1,2-α-thiomannobioside was ring flipped and distorted to the $^3S_1$ conformation suggesting that in this case it is mechanistically-relevant. Indeed, other complexes, notably with mannoimidazole in a $^4H_4$ conformation, and kifenunisine in a $^1C_4$ conformation, as well as subsequent QM/MM analysis of the FEL of α-mannose ‘on-enzyme’. collectively support the $^3S_1 \rightarrow ^2H_4 \rightarrow ^1C_4$ pathway for that enzyme.

In contrast, as discussed earlier, the 1,2-α-thiomannobioside complex observed on family GH92, as observed here for GH125, was also observed undistorted and again silent to conformational pathways; in that case distortion of enzyme-bound mannoimidazole to a boat conformation allowed assignment of a $^0Q_2 \rightarrow ^2B_2 \rightarrow ^2S_2$pathway for that enzyme. This combined work highlights the predictive power of computational methods to use preliminary enzyme-ligand complexes to explore conformational space and generate testable predictions that can provide mechanistic insight using X-ray structural methods. Here these approaches predicted ES distortion for $^3S_1 \rightarrow ^2H_4 \rightarrow ^1C_4$ pathway ($^1C_4$ chair mimic).

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge on the ACS Publications website. Coordinates have been deposited with PDB codes 5M7Y and 5M7I.

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CR, SJW and GJD designed experiments. S A-G performed computational work, AM structural work and PF organic synthesis. GJD, SJW and CR wrote the manuscript.

Notes
The authors declare no competing financial interests.

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REFERENCES
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