Enzymatic Molecular Mechanism of the Human O-GlcNAcase to Design New Inhibitors: A Quantum Mechanical Approach

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In this study the catalytic mechanism of O-glycoprotein 2-acetamino-2-deoxy-\(\beta\)-D-glucopyranosidase (O-GlcNAcase) has been studied by using density functional theory (DFT) at the B3LYP level and split-valence 6-311G** basis set. The results indicate that the reaction path takes place in a two-step mechanism. In the first step, Asp\textsuperscript{174} polarizes the 2-acetamido group, which makes it act as a catalytic nucleophile to form the oxazoline intermediate. In the following step, Asp\textsuperscript{175} acts as a general base to promote the attack of one water molecule at the anomeric center to produce the \(\beta\)-hemiacetal product. This reaction path involves two transition states (Ts1 and Ts2) and one intermediate.

Keywords O-GlcNAcase; Inhibitors; Reaction mechanism; QM calculation

INTRODUCTION

O-Glycoprotein 2-acetamino-2-deoxy-\(\beta\)-D-glucopyranosidase (O-GlcNAcase) is the enzyme responsible for the cleavage of the \(\beta\)-O-linked GlcNAc residues from the serine or threonine residues of nucleocytoplasmic proteins.\cite{1-6} At the sequence level, the glycoside hydrolase domain is a member of the family GH 84 of the CAZY classification.\cite{7,8} Recently, perturbations in the regulation of
the O-GlcNAc have been related to type II diabetes and neurodegenerative disorders such as Parkinson’s and Alzheimer’s disease.\cite{9–13}

O-GlcNAcase is known to act with net retention of an anomeric center configuration, generating the \( \beta \)-anomer of GlcNAc as a product from the \( \beta \)-linked substrate.\cite{14,15} The O-GlcNAcase-catalyzed reaction takes place through a two-step mechanism involving the formation of a transient oxazoline intermediate that is subsequently broken (Sch. 1).\cite{16,17} In this mechanism the \( N \)-acetyl carbonyl group of the substrate, which is activated by an aspartate residue, acts as an intramolecular nucleophile to facilitate catalysis via an oxazoline intermediate. This mechanism is named a substrate-assisted or neighboring group participation catalytic process. Vocadlo and colleagues\cite{14} have elucidated this mechanism by classic Taft analysis. According to this mechanism, in the first step Asp\textsuperscript{174} polarizes the 2-acetamido group to act as a catalytic nucleophile to attack the anomeric center and form the oxazoline ring as an intermediate,\cite{16} while Asp\textsuperscript{175} acts as a general acid to encourage the departure of the leaving group.\cite{16} Then, Asp\textsuperscript{175} acts as a general base to promote the attack of a water molecule to the anomeric center to yield the hemiacetal product (Sch. 1).\cite{18,19} The hypothetical transition state for O-GlcNAcase has been used as a model to design inhibitors of this enzyme.\cite{14,20}

\textbf{Scheme 1:} Proposed mechanism of the reaction catalyzed by O-GlcNAcase.
Small molecule inhibitors of β-N-acetylhexosaminidase have received a great deal of attention for two reasons: (1) as tools for elucidating the role of these enzymes in biological processes and (2) for developing therapeutic interventions with minimal side effects. Since O-GlcNAcylation is regulated by O-GlcNAcase, the modulation of O-GlcNAc levels with small molecule inhibitors of this enzyme should be a suitable strategy for detecting the functions of O-GlcNAcylation in a range of cellular processes. While experimental\textsuperscript{[21–24]} and theoretical\textsuperscript{[25–27]} investigation about the design, synthesis, and characterization of these kinds of molecules have been done, detailed studies of these molecules are desirable.

In this study, we used the quantum mechanical calculations for studying the catalytic mechanism of O-GlcNAcase involving substrate-assisted catalysis. We used two inhibitors, compounds A and B shown in Figure 1, to study the free energy profile for formation of transition states and intermediates through the catalytic mechanism in order to discover the most effective model to design more efficient inhibitors. Determination of the barrier energies and thermodynamic functions through the catalytic mechanism of O-GlcNAcase using high-level calculations will contribute to advance our knowledge on the mechanism of the inhibitors’ action.

**COMPUTATIONAL DETAILS**

All calculations were carried out with the Gaussian program series 2003.\textsuperscript{[28]} Optimization of the geometry was performed employing a hybrid Hartree-Fock density functional scheme, the adiabatic connection method, and the Becke three-parameter with Lee-Yang-Parr (B3LYP) functional\textsuperscript{[29]} of density functional theory (DFT)\textsuperscript{[30]} with the standard 6-311G\textsuperscript{**} basis set. Full optimizations were performed without any symmetry constrains. This level of theory has been shown to give reasonable potential energy surfaces for D-aldo and D-ketohexoses and reduces the basis superposition error.\textsuperscript{[31]} We computed the harmonic vibrational frequencies to confirm that an optimized geometry correctly corresponds to a local minimum that has only real frequencies.

The QST3 procedure was used to search for transition states. All TS geometries were double checked by using IRC and FREQ calculations. In addition,
the thermodynamic properties of all compounds were obtained from frequency calculations at 298.15 K and 1.0 atmosphere pressure. All reported enthalpies were zero-point (ZPE) corrected with unscaled frequencies.

The solvent effects on the conformational equilibrium and contribution to the total enthalpies were investigated with the polarized continuum model (PCM) method\cite{32} at the B3LYP/6-311G** level. Solvation calculations were carried out for water with geometry optimization for this solvent.

RESULTS AND DISCUSSION

Geometry Optimization

The structure of enzyme inhibitors \(A\) and \(B\) were fully optimized by the B3LYP method using the 6-311G** basis set with no initial symmetry restrictions and assuming C point group. The optimized geometry of \(A\) and \(B\) in gas phase was reoptimized by considering the solvent effect (\(\epsilon = 78.9\)) using the PCM method. Tomasi's polarized continuum model defines the cavity as the union of a series of interlocking atomic spheres. The effect of polarization of the solvent continuum is represented numerically.\cite{32}

The results of MD simulation by Brameld and Goddard for hexaNAG substrate bound to the active site of chitinase indicated the substrate conformational distortion during the enzymatic hydrolysis.\cite{33} They proposed that the hydrolysis mechanism of chitinase involves substrate distortion and that the protonation of the linking anomeric oxygen requires a boat conformation for the GlcNAc residue at the binding subsite.\cite{33} Therefore, the first step of the reaction starts with the substrate in the boat conformation.\cite{7,14}

To analyze the substrate distortion, we use the optimized chair conformation of compounds \(A\) and \(B\) as a starting point. The barrier energy between chair and boat conformer is about 3.9 and 10.0 kcal/mol for \(A\) and \(B\), respectively, in the solvent (Fig. 2). This result indicates that both chair and boat conformations are close in energy. Our result is in good agreement with theoretical study using MD simulation by Lameria et al.\cite{26} and experimental data by Macauley et al.\cite{14} that showed the substrate in the boat conformation was the starting point in the reaction path.

A selection of calculated bond distances, bond angles, and dihedral angles are compiled in Table 1. Calculation of vibrational frequencies has confirmed the stationary point with no negative eigenvalue observed in the force constant matrix.

Searching for Reaction Path

According to Scheme 1, it has been proposed that in the first step of the O-GlcNAcase catalytic mechanism Asp\(^{174}\) polarizes the carbonyl group of the
substrate C2 acetamido group for acting as a catalytic nucleophile to form an oxazoline ring. So, in order to find the activation energy for cyclization we used the optimized boat conformer geometry of compounds $A$ and $B$ in the presence of Asp$^{174}$ and Asp$^{175}$ to construct the oxazoline ring and optimized this structure as a transition state (TS1) in gas phase and then in the water solution. The results of the QST3 procedure and frequency calculation with one imaginary frequency confirmed the transition state geometry. The variation energy of this reaction path for $A$ and $B$ is presented in Figures 3 and 4. The energy barrier between the boat conformer and TS1 is about 33.10 and 43.20 kcal/mol for $A$ and $B$, respectively, in the water. The optimized bond distances of C1-O9 and C1-O10 in TS1A are about 2.66 Å and 2.12 Å and in TS1B about 2.72 Å and 2.11 Å, respectively. These bond distance values indicate the C1-O10 bond breaking and the approximation of the 2-acetamido carbonyl oxygen on the

Figure 2: The optimized geometry of inhibitors $A$ (upper) and $B$ (lower) in the chair and boat conformations in solution.
Table 1: Presentation of some structural details of optimized structures of inhibitors A and B in the solvent for chair and boat conformers

| Connected atom | Compound A Chair | Compound A Boat | Compound B Chair | Compound B Boat |
|----------------|------------------|-----------------|------------------|-----------------|
| Bond distance (Å) |                  |                  |                  |                  |
| C1–O9          | 3.13             | 3.78            | 4.28             | 3.85            |
| C1–O10         | 1.40             | 1.45            | 1.42             | 1.48            |
| C1–O13         | —                | —               | —                | —               |
| O10–H11        | —                | —               | —                | —               |
| H11–O12        | —                | —               | —                | —               |
| O13–H14        | —                | —               | —                | —               |
| H14–O12        | —                | —               | —                | —               |
| C1–C2          | 1.54             | 1.53            | 1.54             | 1.54            |
| C1–O6          | 1.42             | 1.39            | 1.40             | 1.37            |
| N7–C2          | 1.46             | 1.45            | 1.47             | 1.45            |
| N7–C8          | 1.37             | 1.37            | 1.36             | 1.37            |
| C8–C9          | 1.22             | 1.22            | 1.23             | 1.22            |
| Bond angle (°) |                  |                  |                  |                  |
| O6–C1–C2–C3    | 110.15           | 113.48          | 111.58           | 114.74          |
| C1–C2–C3–C4    | 110.13           | 111.77          | 107.37           | 111.89          |
| C1–C2–N7–C8    | 113.27           | 111.29          | 107.68           | 110.53          |
| C2–N7–C8–C9    | 123.91           | 122.41          | 130.28           | 122.35          |
| Dihedral angle (°) |                  |                  |                  |                  |
| C1–C2–C3–C4    | -51.46           | 13.04           | -52.68           | -11.08          |
| O6–C1–C2–C3    | 54.27            | -53.92          | 60.06            | -40.42          |
| C1–C2–N7–C8    | -59.57           | -108.13         | -140.25          | -113.72         |
| C8–N7–C2–C3    | 66.01            | 124.97          | -19.33           | 119.59          |

anomeric carbon C1. In addition, the average O10-H11 and H11-O12 (Asp175) values are about 1.62 Å and 1.77 Å in TS1A and 1.01 Å and 1.69 Å in TS1B, respectively, which indicates that Asp175 acts as a general acid to facilitate departure of the aglycone. A detailed analysis of the contribution of Asp174 and Asp175 to form TS1 and TS2 shows that the TS geometry is established with Asp residues through the reaction path. The fact that Asp174 and Asp175 together stabilize all species created along the reaction profile is in agreement with mutagenesis and structural studies reported for O-GlcNAcase from bacterial Clostridium perfringens by Toleman et al.,[34] indicating that Asp residues are the key catalytic residues of O-GlcNAcase.

The geometry of intermediate has been found from the IRC calculation. The result of geometry optimization of intermediate in solvent shows that the average C1-O10 bond distance is about 3.5 Å, suggesting the leaving group departure completely.

According to Scheme 1, Asp175 acts as a general base to promote the attack of a water molecule to generate the hemiacetal product. The geometry of the second transition state in the present of one water molecule and Asp175
Figure 3: Variation of potential energy through the reaction path for inhibitor A.

has been established by using the QST3 procedure and then reoptimized in water. The results of frequency calculation with one imaginary frequency confirm the transition state. According to Figures 3 and 4, the activation energy between intermediate and TS2 is about 10.22 and 16.5 kcal/mol for A and B, respectively, in water. Some electronic structural details of Ts1, INT, and TS2 for A and B are compiled in Table 2. Recently, Vocadlo and coworkers[35] calculated the electrostatic potential surface for PUGNAc and NAG-thiazoline. They found three species along the gas phase reaction path of the cyclization step including O-GlcNAcase and O-GlcNAc: ground state, transition state, and oxazoline intermediate. Our calculated results in a high level of computation provide a more realistic picture and more complete reaction profile through the catalytic mechanism of O-GlcNAcase in solution phase. NAG-thiazoline
and GlcNAcstatin molecules, which are the most potent human O-GlcNAcase inhibitors, have an obvious geometrical resemblance to TS2 and TS1, respectively. In conclusion, to design efficient inhibitors for an enzyme, they must resemble the transition state of the catalyzed reaction as much as possible.

**Calculation of Thermodynamic Functions**

No experimental data of thermodynamic functions such as standard enthalpies of reaction ($\Delta H_{rxn}$) and the standard Gibbs free energies of reaction ($\Delta G_{rxn}$) for both substrates are available. Thus, $\Delta U_{rxn}^o$, $\Delta H_{rxn}^o$, $\Delta S_{rxn}^o$, and $\Delta G_{rxn}^o$ were calculated for both compounds according to the total reaction shown in Scheme 1.
Table 2: Some calculated structural details of TS1, INT, and TS2 for inhibitors A and B in the solvent

| Connected atom | TS1 A | TS2 A | TS1 B | TS2 B | INT A | INT B |
|----------------|-------|-------|-------|-------|-------|-------|
| Bond distance (Å) |       |       |       |       |       |       |
| C1–O9         | 2.66  | 2.04  | 2.72  | 2.04  | 1.57  | 1.57  |
| C1–O10        | 2.12  | —     | 2.11  | —     | —     | —     |
| C1–O13        | —     | 1.96  | —     | 1.96  | —     | —     |
| O10–H11       | 1.62  | —     | 1.01  | —     | —     | —     |
| H11–O12       | 1.77  | —     | 1.69  | —     | —     | —     |
| O13–H14       | —     | 1.81  | —     | 1.81  | —     | —     |
| H14–O12       |       |       |       |       |       |       |
| C1–C2         | 1.52  | 1.51  | 1.48  | 1.51  | 1.54  | 1.54  |
| C1–O6         | 1.45  | 1.51  | 1.31  | 1.51  | 1.33  | 1.33  |
| N7–C2         | 1.46  | 1.47  | 1.45  | 1.47  | 1.47  | 1.47  |
| N7–C8         | 1.38  | 1.46  | 1.39  | 1.46  | 1.31  | 1.31  |
| C8–C9         | 1.22  | 1.22  | 1.22  | 1.22  | 1.29  | 1.29  |
| Bond angle (°) |       |       |       |       |       |       |
| O6–C1–C2      | 110.93| 111.68| 115.97| 111.68| 119.35| 119.35|
| C1–C2–C3      | 106.25| 110.92| 105.15| 110.92| 113.70| 113.70|
| C1–C2–N7–C8   | 114.81| 110.12| 118.60| 110.12| 100.70| 100.70|
| C2–N7–C8      | 125.09| 110.39| 125.92| 110.39| 111.32| 111.32|
| Dihedral bond (°) |       |       |       |       |       |       |
| C1–C2–C3–C4   | −51.16| −54.93| −53.09| −54.93| −40.56| −40.56|
| O6–C1–C2–C3   | 60.24 | 55.44 | 59.99 | 55.44 | 27.37 | 27.37 |
| C1–C2–N7–C8   | −13.95| 9.73  | −0.81 | 9.73  | −21.31| −21.31|
| C8–N7–C2–C3   | 106.47| 131.92| 119.86| 131.92| 99.40 | 99.40 |

Total enthalpies of the studied species X, H(X), at the temperature T are usually estimated from Equation (1) shown below:

\[
H(X) = E_0 + ZPE + E_{\text{trans}} + E_{\text{rot}} + E_{\text{vib}} + RT
\]

where \(E_0\) is the calculated total electronic energy; ZEP stands for zero-point energy; and \(E_{\text{trans}}, E_{\text{rot}},\) and \(E_{\text{vib}}\) are the translational, rotational, and vibrational contributions to the enthalpy, respectively. Finally, RT represents the PV-work term and is added to convert the energy to enthalpy.

The standard enthalpy change of the reaction \(\Delta H^{\circ}_{\text{rxn}}\) is given as:

\[
\Delta H^{\circ}_{\text{rxn}} = [H^{\circ}_{\text{product}}] - [H^{\circ}_{\text{reactant}}]
\]

Table 3: Calculated thermodynamic properties for inhibitors A and B in the reaction in water

|                  | \(\Delta U\) (kcal/mol) | \(\Delta H\) (kcal/mol) | \(\Delta S\) (kcal/mol) | \(\Delta G\) (kcal/mol) |
|------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| Compound A       | −73.99                  | −74.31                  | −0.002                  | −73.30                  |
| Compound B       | −15.21                  | −15.53                  | −0.003                  | −15.40                  |
Table 4: Calculated thermodynamic functions (in kcal/mol) of inhibitors A and B through the reaction pathway in the solvent

|               | Compound A | Compound B |
|---------------|------------|------------|
| $\Delta U^\ddagger_1$ | 33.10      | 43.22      |
| $\Delta G^\ddagger_1$ | 45.61      | 32.40      |
| $\Delta H^\ddagger_1$ | 33.69      | 43.80      |
| $\Delta S^\ddagger_1$ | –0.04      | –0.50      |
| $\Delta U^\ddagger_2$ | 10.22      | 16.50      |
| $\Delta G^\ddagger_2$ | 13.79      | 20.07      |
| $\Delta H^\ddagger_2$ | 10.81      | 17.09      |
| $\Delta S^\ddagger_2$ | –0.01      | –0.01      |

Table 4: Calculated thermodynamic functions (in kcal/mol) of inhibitors A and B through the reaction pathway in the solvent

in which total standard enthalpies of the studied species, at the temperature $T$, are estimated from Equation (1).

Similarly, $\Delta S^\circ_{\text{rxn}}$ could be obtained by:

$$\Delta S^\circ_{\text{rxn}} = [S^\circ_{\text{product}}] - [S^\circ_{\text{reactant}}]$$  \hspace{1cm} (3)

According to thermodynamic equation, $\Delta G = \Delta H - T\Delta S$, the $\Delta G^\circ_{\text{rxn}}$ was calculated.

The calculated thermodynamic properties in the total reaction for both A and B are reported in Table 3. The negative values of $\Delta H^\circ_{\text{rxn}}$ and $\Delta G^\circ_{\text{rxn}}$ for both inhibitors indicate the exothermicity and spontaneity of the desired reaction for both inhibitors. The more negative values of $\Delta H^\circ_{r}$ and $\Delta G^\circ_{r}$ are found in the case of inhibitor A rather than inhibitor B. Therefore, from thermodynamic properties it is concluded that the inhibitory activity is affected by the kind of substitution on anomeric carbon. These results show the role of the R group in the biological activity of these inhibitors.

The calculated activation free energies, $\Delta G^\neq$, for Ts1 and Ts2 are 45.6 and 13.79 kcal/mol for A and 32.40 and 20.07 kcal/mol for B, respectively, as shown in Table 4. On the basis of these results, it is suggested that the glycosidic bond cleavage in the first step could be the rate-limiting step of the reaction.

CONCLUSION

Quantum mechanical calculations have been applied to study the catalytic mechanism of O-GlcNAcase to hydrolyze O-GlcNAc. The energy profiles for inhibitors A and B indicate the formation of oxazoline intermediate in the O-GlcNAcase-catalyzed reaction occurring in a stepwise mechanism. In the first step, Asp$^{174}$ polarized the 2-acetamido group of the substrate, making it act as a nucleophile to attack the anomeric center and displace the leaving group, while Asp$^{175}$ acts as a general acid to facilitate departure of the leaving group.
group. It is noticeable that O-GlcNAc is distorted to form a boat conformation before nucleophile attack of the 2-acetamido group. According to the calculated potential energy surface, two transition states and a high-energy intermediate between them has been found in the catalyzed pathway. These results suggest that the most potent inhibitors for OGlcNAcase such as NAG-thiazoline, PUGNAc, and GlcNAcstatin derivatives could be considered as transition state mimics. The results are expected to be helpful to explain some of the experimental observations. In addition, our theoretical study not only is valuable to gain insight into the reaction but also provides details about the molecular mechanism, electronic, structural, and thermodynamic information of the species appearing along the enzymatic reaction pathway, which has great value for the design and development of new inhibitors.

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